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&
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Evaluation of a tetravalent DNA vaccine against dengue:
Integrating biochemical studies on Dengue virus envelope protein to a
Domain-based antigen design.

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A Thesis submitted in fulfilment of the requirements of the Faculty of Life Sciences
of the Open University (UK) for the Degree of Doctor of Philosophy.

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**Trieste, Italy
December, 2016**

"It's a dangerous business, Frodo, going out your door. You step onto the road, and if you don't keep your feet, there's no knowing where you might be swept off to".

J.R.R. Tolkien

Para Mamina, otra vez...

Dengue virus (DENV) is among the most important mosquito-borne human pathogens worldwide. Concerns regarding the effectiveness of Dengue vaccines together with emergence of Zika virus (ZIKV) have reignited the interest for new vaccines using alternative approaches. Given that the envelope glycoprotein (E) is the main target of neutralizing antibodies, it has been used as the antigen of choice for vaccine development efforts.

Here we present a detailed analysis of factors involved in the expression, secretion and folding of the E ectodomain from all four DENV serotypes and ZIKV in mammalian cells. Our data demonstrate that E domains II and III (DII and DIII) are important for proper E folding and stabilization of soluble dimers, respectively. In addition, we show that successful covalent stabilisation of E dimers, and E folding in general is strongly dependent on temperature but not on PrM co-expression, and that DENV and ZIKV E proteins can form heterodimers and assemble into mosaic viral particles.

Our findings also show that antigen secretion determines the efficiency of DNA vaccines. Based on this, we developed a novel DNA gene-gun immunisation strategy using an engineered version of DIII fused to the CH3 domain of the IgG H chain, which is efficiently secreted from transfected cells and induced strong antibody responses that neutralise all DENV serotypes. The antibody responses were stable over long periods of time and different tetravalent formulations of the vaccine showed induction of neutralising antibodies against all four dengue serotypes as well.

Finally, our results also indicate that the polyclonal antibody responses against DI/DII are highly cross-reactive, poorly neutralising and promote ADE towards all DENV serotypes, ZIKV, WNV and YFV. Conversely, anti-DIII antibodies are type-specific, with no ADE towards related flaviviruses, and with strong neutralisation activity restricted only to DENV.

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INTRODUCTION

1. OVERVIEW ON THE HISTORY OF DENGUE

1.1. History of Dengue Disease

The historic footprint of mosquito-borne dengue disease became a subject of study after the isolation of viral agent in suckling mice by Sabin and Schlesinger in 1944^{1,2}.

Leading to this discovery, the data accumulated since the beginning of the 1900's was pivotal to understand the behaviour of the disease starting with the description of its transmission by *Aedes aegypti* in 1906^{1,3}, the demonstration that the etiologic agent had virus-between mosquito and humans^{1,3,4}.

Dengue viruses have been impacting humans for centuries. The first epidemic report of a disease with the clinical and epidemiological presentation of dengue was made by Benjamin Rush in Philadelphia, United States, in 1780 with similar epidemic reports of dengue-like illnesses in Africa, Asia and Europe also happening during that period^{5,6}. Although the aforementioned records describe the characteristics of dengue with precision, the oldest recorded illnesses with dengue-like symptoms date back to the 3rd, 7th and 10th century in China for a disease called "water poison" due to its association with near-water environments^{5,7}. Thus, dengue disease reached global distribution and pandemic proportions before the wake of 1800, which coincides with the increase in sailing and ship-mediated global commerce⁷.

A second wave of pandemics with the same clinical features was registered across the world during most of the 19th century and the beginning of the 20th, where a commerce-driven explanation for the spreading of the disease was first made by recognizing dengue as a condition typical of seaports and coastal areas that travelled inland along the rivers^{1,3,5,7}. Parallel observations regarding not only the introduction and spreading, but also the domestication of mosquitos in America and other regions of the world⁸, laid the ground for the discovery of *A. aegypti* as vector of the disease. The sum of all these factors altered the behaviour of dengue allowing the establishment of endemic regions across the Southeast Asia region before 1950⁷.

It was World War II that created the conditions for a new relationship between dengue and humans: the movement of troops and war materials from the coasts to the inlands in Asia and the Pacific, along with the huge environmental changes provoked by the war effort, completely transformed the ecological and epidemiological scenery of dengue, allowing the spread of the vector and, consequently, increasing the transmission and distribution of the disease, setting the stage for the beginning of the global dengue pandemic^{5,9}. By the end of the war, many regions in Asia reach hyperendemic status with active co-circulation of all four viral serotypes. Additionally, the demographic changes induced by the war and the economic growth that followed in many Southeast Asian countries, led to uncontrolled urbanization where inadequate housing and management of both water supply and sewage allowed further expansion and domestication of the vector,

increasing the rates of transmission and facilitating the dispersal of the virus across the region^{5,7}. It was during this period that the first documented epidemics of dengue haemorrhagic fever occurred in the Philippines and Thailand. Within 20 years, dengue spread throughout the region becoming a leading cause of children's hospitalization and death by the 1980's^{5,10,11}.

Epidemiological changes were most dramatic in America where, following World War II, dengue epidemics appeared to be under control due to the efforts of the Pan-American Health Organization to control yellow fever disease, which led to the eradication of *A. aegypti* from most of Central and South America^{5,7,11}. After the discontinuation of the programme in the 1970's the vector was reintroduced in the region where it regained its previous geographic distribution^{6,12}. By the end of the 1980's, the region was experiencing major epidemics that worsen after the introduction of new serotypes and strains; ten years later, many countries evolved from non-endemicity to hyper-endemicity with active circulation of all serotypes in the continent^{5,13-15}.

By the end of the 20th century, dengue viruses reached worldwide distribution within the tropics and caused more illness and death than any other arthropod-borne virus in humans^{5,6,12}.

1.2. Etymology of “Dengue”

The origins of the word dengue are not clear. A dengue-like disease that originated in West Africa during 1870 and spread to India the following year, led to the discovery that the Swahili word for the illness was *ki-dinga-pepo* that means “cramp-like seizure caused by an evil spirit”³. Historic records show that the term *dyenga* had been used to describe a similar disease breakout during the 1820's, however, the earliest record of the word *dengue* comes from 1800's records of the Spanish court, so it's possible that the Swahili word *dinga* may have actually originated from Spanish¹⁶.

There is substantial evidence indicating that the disease, was spread along the Caribbean and the West Indies as a result of the African slave trade¹⁶. In Cuba, the disease was identified again with the Spanish word *dengue* during an 1828 outbreak of a similar condition, while word *dandy* was used in the West Indies. In both cases, the meaning of the word indicates “fastidiousness” and seem to reference the stiffness and joint pain associated with the disease¹⁶. After being used within the Cuban medical records, the word dengue was established in medical literature³.

2. THE DENGUE VIRUS

2.1. Classification: General perspectives of Flaviviruses

Dengue viruses are members of the *Flavivirus* genus that together with the genera *Hepacivirus*, *Pestivirus* and *Pegivirus*, form the taxonomical family known as *Flaviviridae*,

one of the largest families of viral pathogens responsible for causing severe diseases and mortality in humans and animals^{1,17}.

Initially, flaviviruses (along with the pestiviruses) were considered members of the family *Togaviridae*; however, deeper analysis into the biology, genetics and structure of these viruses justified their reclassification into the new *Flaviviridae* family¹. The *Flavivirus* genus is composed of more than 70 viruses, most of them associated with human diseases including some of the most important human pathogenic viruses in the world besides dengue: Zika virus (ZIKV), Yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV) (Fig. 1). Species demarcation criteria within the genus include: nucleotide and amino acid sequences, antigenic characteristics, geographic association, vector association, host association and ecological characteristics¹⁸.

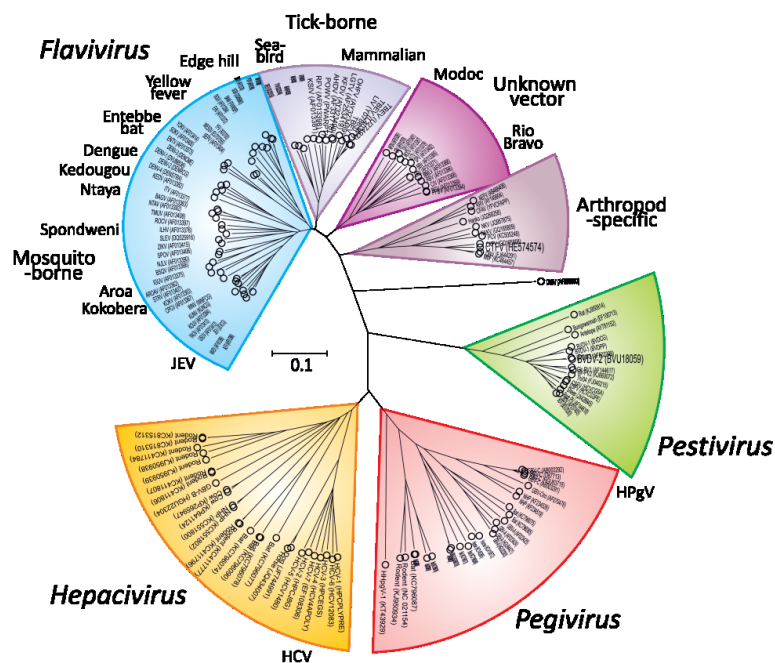


Figure 1. Phylogeny using the conserved sequences in the RNA-dependent RNA-polymerase of flaviviruses (Reprint from reference 18).

Structurally, flaviviruses virions are around 50nm in diameter, spherical in shape and consist of a spherical ribonucleoprotein core surrounded by a lipoprotein envelope with small projections. Each virion's dry weight consists roughly of 6% RNA, 66% proteins, 17% of lipids derived from the host cell membrane and about 9% of carbohydrate with a composition and structure that varies depending on the host cell^{1,17,18}. Regarding their physicochemical properties, flaviviruses are stable at an alkaline pH between 8.0 and 8.4 showing high sensitivity to acid environment¹. Additionally, these agents are rapidly inactivated at temperatures above 50°C, ultraviolet light, gamma irradiation, organic solvents, detergents, alcohol and hydrogen peroxide^{1,18}.

Genome-wise, the *Flaviviridae* family are characterized by carrying single-stranded, positive-sense RNA genomes between 9.5 kb and 12.5 long. Members of the *Flavivirus* genus in particular, possess the distinguishing feature of having a type I cap (m⁷GpppA-m²) at the 5' end of their genome^{1,18}.

On the basis of their ecology, flaviviruses belong to the group termed “arbovirus”. Arboviruses (Arthropod-borne viruses) are a taxonomically diverse group biologically transmitted among their vertebrate hosts by haematophagous arthropod vectors like mosquitos, ticks, midges and biting flies^{7,19,20}. By definition, biological transmission implicates that arboviruses must replicate in the vector before being transmitted to the vertebrate host, this means that the transmission cycle requires viral replication within the arthropod as opposed to mechanical transmission which can occur without²⁰. Biological transmission can be vertical or horizontal. This last mode of transmission, mainly involves transmission from vector to vertebrate host during blood feeding and is the most common route of infection for the majority of arboviruses²⁰.

Besides *Flaviviridae*, the arboviruses include a very diverse group are RNA viruses taxa like *Bunyaviridae*, *Orthomyxoviridae*, *Reoviridae* and *Rhabdoviridae*; the fact that these groups show a wide variety of RNA genomes and replication strategies indicates that the arthropod-borne transmission trait has arisen many different times during the evolution of RNA viruses¹⁹.

Dengue viruses are highly restricted in their natural vertebrate host range, utilizing primates, almost exclusively humans, as reservoir and amplification host⁷; therefore, it's no surprise that among all arboviruses, Dengue viruses are the most important human pathogens¹⁹.

2.2. Origin and Evolution of Dengue Virus

All Dengue viruses are classified within the same antigenic complex in the genus *Flavivirus*; the inclusion in this group was based on antigenic cross-reactivity with other flaviviruses and confirmed with genomic organisation and sequence homology studies^{7,21}. Evolution, dispersal patterns and epidemiological traits of flaviviruses originated in a combination of barriers imposed by the nature of their vectors and the ecology in which they have developed²². However, it was only after the development of detailed molecular techniques that the evolutionary history of dengue virus started to be unveiled^{23,24}.

Dengue virus serogroup comprises four different but closely related serotypes (DENV1, DENV2, DENV3 and DENV4) defined originally on the basis of antigenic cross-reactivity of the virus envelope glycoprotein (E) with neutralising antibodies from sera of infected volunteers in diverse serological tests^{4,25}. The four dengue serotypes were later confirmed by nucleic acid sequencing and also by ecologic, phylogenetic and evolution studies^{1,7,23}. Genetically, the four serotypes are diverse and share between 60-75%

aminoacid identity^{26,27}, while isolates within the same serotypes have only an average of 3% aminoacid divergence (roughly 6% at the nucleotide level)²⁸ which has been associated with fitness and virulence determinants²⁶. However, given the lack of resolution within the flaviviral phylogenetic tree, the closest relatives of dengue virus cannot be identified with any certainty, which renders the reconstruction of dengue virus origin a difficult task.

The strongest data on dengue virus origin concerns the identification of dengue's last animal reservoir before evolving into a human disease. Researchers hypothesized that the actual version of the human endemic virus initially evolved from sylvatic strains that utilized non-human primates (NHP) as hosts with environmentally-associated *Aedes spp.* mosquitoes serving as vectors^{7,15,23}.

This assumption of Dengue virus being originally zoonotic is supported by some key observations on the existence of sylvatic strains of DENV2 and DENV 4 in Africa that fell basal to the human-isolated strains within their serotype in phylogenetic studies^{7,29,30}. The hypothesis of a sylvatic Dengue virus origin was further supported by performing phylogenetic analysis after sequencing the *E* gene of DENV 1, 2 and 4 sylvatic strains from Malaysia and Africa^{30,31}. As before, the sylvatic strains of the virus were distinct from those isolated from humans and vectors in an endemic scene. Although a sylvatic strain of DENV3 has not been isolated, the presence of antibodies against this serotype in Malaysian primates points to the existence of a sylvatic DENV3 cycle as well³⁰. Thus, there is strong data supporting the sylvatic nonhuman-primate origin of dengue virus and that transmission to humans occurred later on for all four serotypes^{7,23}.

Less clear is where Dengue virus originated. Gaunt and others²² proposed an African origin based on the available data for other mosquito-borne flaviviruses, implying that the whole clade may have originated there; moreover, *A. aegypti* is also believed to have originated in Africa^{8,23}. Conversely, the fact that there is abundant evidence for the sylvatic presence of all 4 serotypes in Asia suggest an Asian origin rather than African²³.

By estimating rates of nucleotide substitution, various investigators have been able to draw some light into the time-scale evolution of dengue virus³². Using the *E* gene from dengue, several groups reached the common conclusion that the endemic variants of DENV2 diverged first from their sylvatic ancestor close to 300 years ago, with the origin of the whole dengue virus group tracing back to a remarkable recent date of about 1000 years which is in accordance with the first reports of the disease³²⁻³⁵. Therefore, the history of dengue seems to be a recent one in which up to a few hundred years ago the virus was mainly a zoonotic disease causing only rare outbreaks in humans. Transmission among humans was sustainable only after the demographic changes of the last centuries²³.

Regarding the origin of the serotypes, most evidence gathered so far favours an independent evolution hypothesis in which the virus originally separated into different groups because of geographic or ecological reasons, in such a way that the 4 serotypes evolved independently from each other in four separate dengue epidemics with repeated adaptations to new vectors and vertebrate hosts^{5,7,23}.

2.2.1. Dengue virus serotype 1 (DENV1)

This serotype was first reported in 1943 in French Polynesia and Japan, with reports increasing constantly over time in Asia from the late 1950's. It was not reported in America until 1977 in Barbados, Cuba and Puerto Rico; since then, occurrences increased persistently across the region peaking in 2006. In Africa, the serotype was first reported in 1984 and has been sporadically reported ever since³⁶.

Phylogenies based on the complete sequence of DENV1 *E* gene have confirmed the existence of 5 different genotypes: i) genotype I from strains of Southeast Asia, China and East Africa; ii) genotype II representing the 1950's-1960's strain from Thailand; iii) genotype III for the Malaysian sylvatic strain; iv) genotype IV representing the strains of the West Pacific and Australia and v) genotype V grouping all the American, African and remaining Asian strains^{7,37,38}.

2.2.2. Dengue virus serotype 2 (DENV2)

This serotype was first reported in Papua New Guinea in 1944 followed by recurrent and increasing reports in Southeast Asia and the Pacific islands since then. In Africa and America, this serotype was first recorded in the 1960's with frequent reporting of the serotype ever since³⁶.

5 genotypes of DENV2 are now recognized based on complete *E* gene sequences: i) Asian genotype I representing strains from Asia and the Pacific islands; ii) the cosmopolitan genotype that groups strains from all over the world; iii) the America genotype representing strains from Latin America and older strains from the Caribbean and India; iv) the Southeast Asia/American genotype representing strains collected from Thailand, Vietnam and America over the last 20 years; and v) the sylvatic genotype of the strains collected mainly from forest mosquitos and monkeys in Africa and Asia^{7,29}.

2.2.3. Dengue virus serotype 3 (DENV3)

First reported in 1953 in the Philippines and Thailand, DENV3 has been continuously reported in Asia since then. In America, it was first reported in Puerto Rico in 1963 with the majority of American countries not reporting the serotype until the late 1980's. In Africa, only a small number of cases have been reported since the first reports in 1984³⁶.

Current DENV3 phylogenies confirm the existence of 4 real genotypes and one theoretical: i) genotype I, representing isolates from the Pacific Islands; ii) genotype II that groups strains from Thailand, Vietnam and Bangladesh; iii) genotype III represents the strains from Africa and some Indian isolates; and iv) genotype IV representing all American strains. The theoretical genotype V is reserved for sylvatic strains that have not been isolated in the case of Dengue 3 but are believed to exist in Malaysia⁷.

2.2.4. Dengue virus serotype 4 (DENV4)

As for serotype 3, this variant was first reported in 1953 in the Philippines with yearly reports of cases since then. It was not encountered in America until 1981 with continuous reports occurring since³⁶.

Phylogenies for this serotype delineate 4 genotypes: i) genotype I representing strains from Thailand, Philippines, Sri Lanka and Japan; ii) genotype II grouping strains from the Pacific and America; iii) genotype III representing recent Thai strains; and iv) genotype IV that includes the sylvatic strains from Malaysia^{7,38}.

Although in need of further studies and official recognition, there seems to be evidence pointing towards the discovery of a fifth DENV serotype. This new isolate taken from the 2007 dengue outbreak in Malaysia's Sarawak state, was initially thought to be a strain of DENV4, however, the virus did not respond to diagnostic tests as expected. After sequencing its entire genome, it became clear that this virus occupied a new branch in the dengue phylogenetic tree as a new fifth serotype. It was later determined that the antibodies elicited by this strain differed substantially from those obtained after an infection with the other serotypes. Nevertheless, the implications of this event in public health are still unclear because there is no evidence of a sustained transmission cycle of this new serotype in humans, with researchers suspecting that the virus is still contained in sylvatic circulation among macaques³⁹. This report serves as a perfect example for the intense diversification pattern of Dengue viruses.

In general, *Flavivirus* evolution and epidemiology is largely determined by the ecological needs of their arthropod vector and vertebrate host. This type of horizontal biological transmission implicates that these viruses, have to replicate alternately in very different environments⁷. The selective pressures that come from adapting to two very disparate hosts in order to ensure transmission might explain the paradox that, despite the significant potential for sequence changes in RNA viruses mainly because of their error prone RdRp, the consensus sequences of most arboviruses show a very high genetic stability^{7,19}. This implies that, arboviruses requirement for replication in divergent hosts may impose evolutionary constraints in which only mutations that are either beneficial or neutral in both hosts become fixed.

2.3. Viral Structure

Dengue virions have a diameter of $\approx 500\text{\AA}$ and consist of a proteic capsule containing a single molecule of the viral RNA genome inside a host-derived lipid membrane¹⁷. Flaviviral genomes code for 3 structural proteins (the capsid protein (C), the membrane protein (M) and the envelope protein (E)) that ensemble together into viral particles: the envelope protein and the membrane protein form a glycoprotein-based outer shell that's embedded in the lipid membrane while the capsid protein surrounds the viral genome forming the nucleocapsid at the viral core⁴⁰.

As pictured in Fig. 2, cryoelectron microscopy (CryoEM) studies have shown that mature particles have a relatively round, smooth surface with an icosahedral structure formed by asymmetric units of three E-M heterodimers^{41,42}. The highly ordered icosahedral scaffold of the viral surface consists of 180 E molecules that are distributed in 30 rafts of 3 parallel dimers organized in a herringbone pattern (Fig. 2a and 2b)^{3,40,43}. Density analyses show that the viral particle is actually composed of a series of spherical shells (Fig. 2c) starting with the shell formed by the E protein on the viral surface; right underneath lies a M protein shell that is in close contact with the layer formed by the lipid membrane and the transmembrane domains of E and M; together, these three layers compose the viral envelope⁴³. The nucleocapsid shell is inside the lipid membrane and appears to have a disordered structure with only random contacts with the inner layer on the envelope⁴⁴.

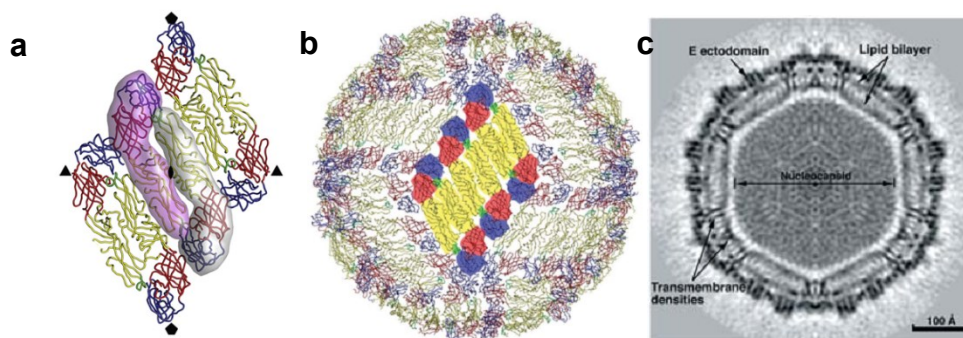


Figure 2. Structure of a mature dengue virus. a) Raft organization of three parallel E homo-dimers. b) Proposed structure for the mature dengue virion highlighting the packaging of the E protein rafts in a herringbone pattern. c) A central cross-section of dengue virus showing the different layers that compose the viral particle (Reprint from references 42, 40 and 44).

The CryoEM structures of the different DENV serotypes have been calculated at different resolutions with superposition analysis showing no significant structural differences among them or among other flaviviruses. There are, however, important variations regarding the electric charge distribution on the surface of the E protein that provoke slight changes on the exposed surface of the virus^{41,43,45-47}.

Early antibody binding studies on the E protein were contradictory with the first structural reports of the virus in the sense that several neutralising antibodies tested on

the viral surface, bound epitopes that were not exposed on the mature virions⁴⁸. These observations are explained by a model called viral “breathing”, in which the E proteins that cover the viral surface are in a continuously dynamic state that involves slight changes in their configuration and arrangement^{48,49}. In addition, recent data published independently by Zhang *et al* and Fibriansah *et al*, indicate that dengue virions suffer further structural variation at temperatures above 33°C, in which the envelope expands (Fig. 3) and increased exposure of E, theoretically favouring viral binding and entry, which has significant implications regarding treatment and vaccine development⁵⁰⁻⁵².

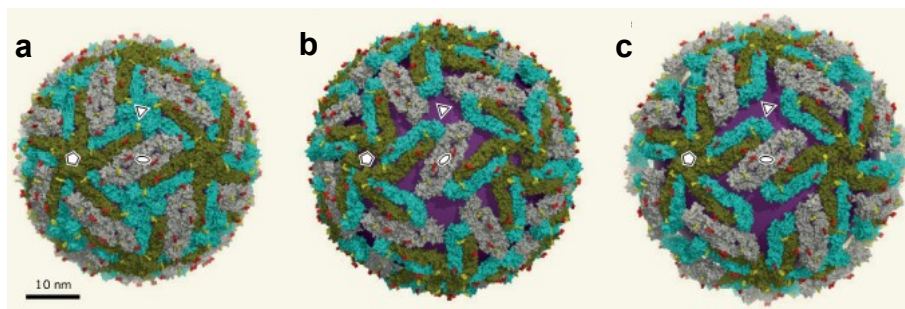


Figure 3. Effect of temperature on viral structure. **a)** The mature and smooth dengue viral particle at 28°C showing all the typical characteristics of classic dengue virion descriptions. **b)** and **c)** The structural models proposed by Zhang *et al*⁵¹ and Fibriansah *et al*⁵⁰, respectively, depicting the structural changes adopted by the E protein at temperatures above 33°C (Reprint from reference 52).

2.4. Dengue virus genome structure

Like all other flaviviruses, the dengue virus genome, is a single-stranded, positive sense RNA molecule of approximately 11 Kb^{18,53}. Characteristically, viruses belonging to this genus carry a type I cap ($m^7GpppAmpN_2$) at the 5' end followed by a conserved AG sequence while the 3'end lacks the typical poly-A tail of cellular messenger RNAs^{3,17,54}.

The flaviviral RNA genome acts as the messenger RNA for translation of the single open reading frame (ORF) encoding a large, ≈ 3440 aminoacids-long, precursor polyprotein that is cleaved and processed post- and co-translationally by viral and cellular proteases into the 10 mature viral proteins^{3,53}. However, unlike cellular mRNAs, which contain only translational regulatory motifs, DENV genomes have evolved to contain a variety of signals and regulatory regions that act at different stages of the viral life cycle⁵⁵. These regulations are mediated by RNA sequences and secondary structures present in the translated and untranslated regions (UTRs) of the molecule that function as promoters, enhancers and repressors of translation, transcription, replication and encapsidation^{17,54,56}. The sequence, localization and predicted secondary structures of these RNA elements are shown in Fig. 4.

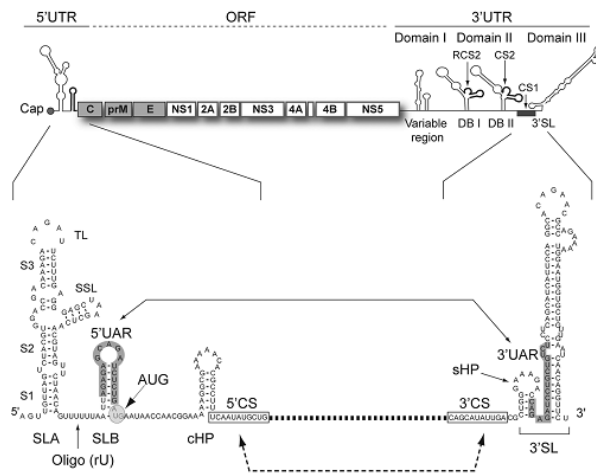


Figure 4. Schematic representation of Dengue virus genome with the sequences and predicted secondary structures of the 5' and 3' untranslated regions (Reprint from reference 56).

Dengue virus 5'UTR sequence is between 95 and 101 bases long depending on the serotype and contains two distinct RNA domains⁵⁶. The first domain is close to 70 nucleotides long and folds into a large stem-loop (SLA) composed of 3 helical regions (S1, S2, and S3), a side stem-loop (SSL) and a top loop (TL). The SLA domain has been proposed to function as a promoter to activate viral RdRp^{54,57}. The second 5'UTR domain is a short stem-loop (SLB) which contains specific signals allowing the long-range RNA-RNA interactions required for replication⁵⁸⁻⁶⁰. Additionally, these two domains are separated by an oligo(U) spacer sequence which allows proper function of both stem-loops during replication^{56,61}. Within the coding sequence of dengue virus genome, there's a stable RNA hairpin (cHP) inside the capsid gene after the translation initiation codon, that is required for viral RNA replication⁶².

The ≈450 nucleotide 3'UTR includes several conserved sequences and structures that play important roles in viral replication and translation⁷. Domain I is the most variable region of the 3'UTR (VR) and is located immediately after the stop codon^{54,63}. Domain II is more or less conserved and includes a characteristic duplicated dumbbell structure (DB1-DB2) containing conserved sequences (CS2 and RCS2) present in all mosquito-borne flaviviruses^{55,56}. Studies have confirmed that both domains participate in the regulation of RNA synthesis and translation as enhancers^{64,65}. Domain III is the most conserved region of the 3' UTR and participates in the regulation of RNA replication⁶⁶.

Another conserved element in the flaviviral genome is the presence of inverted complementary sequences that mediate RNA-RNA long range interactions leading to a cyclization of the viral ARN, an essential event during viral replication⁵⁸.

2.5. Proteins encoded by the virus

As shown in Fig. 5, the flaviviral genome codes for 3 structural proteins (C-PrM-E) that are contained in the N-terminal portion of the polyprotein, and 7 non-structural

proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) that are coded in the remaining C-terminal region⁶⁷. As their names indicate, the structural proteins are components of the mature viral particle whereas the non-structural proteins are presented only in the infected cells to fulfil roles in the viral life cycle^{3,17,68}.

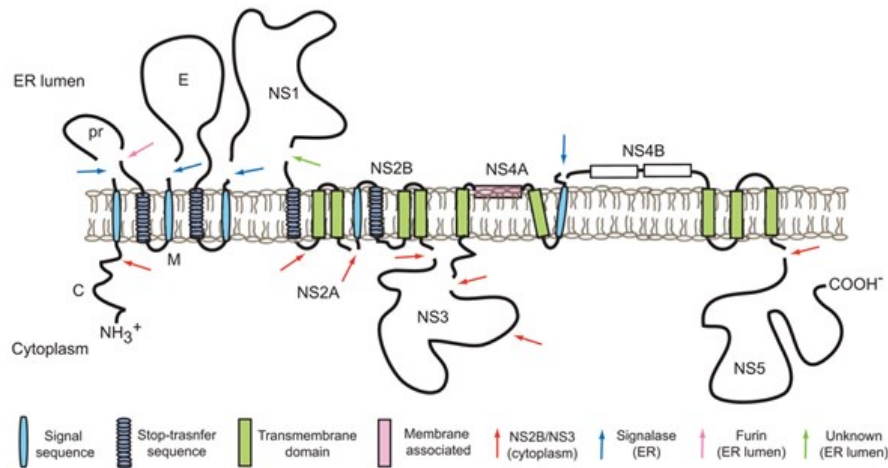


Figure 5. Membrane topology of dengue virus polyprotein. The viral RNA is translated at the ER membrane into a large polyprotein with different membrane, cytosolic and luminal domains that is processed by different proteases indicated by the differently coloured arrows to render the mature viral proteins (Reprint from reference 67).

The genomes of flaviviruses are among the smallest within human pathogens with a maximum capacity of close to a dozen proteins⁶⁹. In spite of these, flaviviruses must coordinate all the steps involved in the production of new viral particles while dealing with the immune system attempts to stop viral replication and destroy infected cells. As a consequence, most viral proteins, including the structural ones, have several functions^{17,69}.

2.5.1. Structural Proteins

2.5.1.1. Capsid Protein (C)

Known also as core protein, the capsid protein is a highly basic protein of 12kDa that is essential for encapsidation of the genome forming the viral nucleocapsid structure together with the genomic RNA molecule⁷.

Flavivirus capsid proteins share a similar distribution of basic aminoacids which leads to an overall conserved structure and charge distribution⁷⁰. Each protein contains 4 alpha-helices, the first three forming the protein's core and the last one extends away on the surface forming a highly basic domain that is proposed to interact with the viral RNA^{71,72}. As shown on Fig. 6, on the opposite side of the molecule there's a largely hydrophobic domain formed by helices 1 and 2, that behaves as a hydrophobic signal sequence allowing interactions between the protein and the cell membranes which has been proposed as fundamental for viral assembly^{17,70,73}.

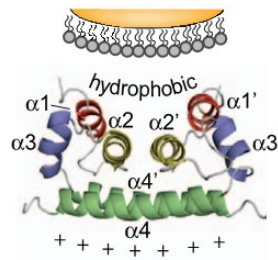


Figure 6. Ribbon diagram for the dimeric structure of dengue virus capsid protein (Reprint from reference 73).

The capsid protein is also involved in a variety of non-structural roles during the viral life cycle that may include RNA replication, regulation of translation, modulation of infectivity, cellular environment and cell survival⁷³⁻⁷⁵.

2.5.1.2. Precursor peptide and membrane protein (PrM)

Upon translation, the membrane protein is the first viral protein to be translocated into the endoplasmic reticulum (ER). Initially, the protein is synthesised as a glycosylated, 26kDa precursor named pre-membrane protein (PrM) which consist of an N-terminal precursor (Pr) domain followed by the M protein at C-terminus, with a sequence coding for a furin cleavage site between them^{17,76}. Structurally, the Pr domain carries the only glycosylation site and is formed by seven antiparallel β -strands that are stabilized by 3 highly conserved disulphide bridges, while the M protein is basically a linear structure formed by an N-terminal loop followed by an amphipathic alpha-helical stem (MH) that interacts very closely with the lipid layer and two transmembrane helices⁷⁶.

After translation, PrM interacts with the nascent envelope protein and remains bound to its fusion loop during the transport through the Trans-Golgi network. The increasingly acid environment triggers a severe conformational change of the PrM-E interaction provoking the release of Pr from the M protein by furin-mediated cleavage, although Pr remains attached to the E protein preventing premature fusion during viral release^{17,77,78}. Other studies in WNV, have shown that proper folding of E can occur only when is co-synthesised with PrM⁷⁹. As a consequence, it has been generally accepted that PrM assists E's proper folding in a chaperone-like manner, with a significant effect on the generation of infective viral particles^{17,77,79}. Once released from the host cell, the Pr peptide separates from the mature infective particle^{77,80}.

Additionally, Zhang *et al* and Hsieh *et al*, presented data supporting an important role for M's MH domain during viral maturation and assembly, and in the entry process to the host cell^{76,81}.

2.5.1.3. *Envelope protein (E)*

In addition to being the main target for neutralising antibodies, the proteins embedded in the viral membrane of enveloped viruses have essential functions during the virus life cycle as determinants of host range, cellular and tissue tropism, virus-cell attachment, virulence, cellular entry (membrane fusion) and viral assembly^{17,45,82}. In flaviviruses, the E glycoprotein is the major constituent of the viral membrane and is synthesized as a ≈500 aminoacids-long, type I membrane protein¹⁷.

Viral membrane fusion proteins can be classified into three different structural groups. Flaviviral E proteins fall into class II proteins that share a 3-domain structure composed mainly of β -strands with a fusion loop in the central domain that serves as the initiator of the membrane fusion process^{83,84}. In the case of dengue, the E protein folds into an elongated rod-like structure that forms antiparallel dimers, with approximate dimensions of 150Å x 55Å x 30Å, that lay with the long axis of the dimer parallel to the membrane in a herringbone-like configuration that induces a curvature to the structure with the convex face of the dimer facing outward^{45,70}.

As pictured in Fig. 7, the E ectodomain, which involves E's first ≈400 aminoacids, folds into three different structural domains named I, II and III that are discontinuous when compared against the primary structure of the protein^{45,85}.

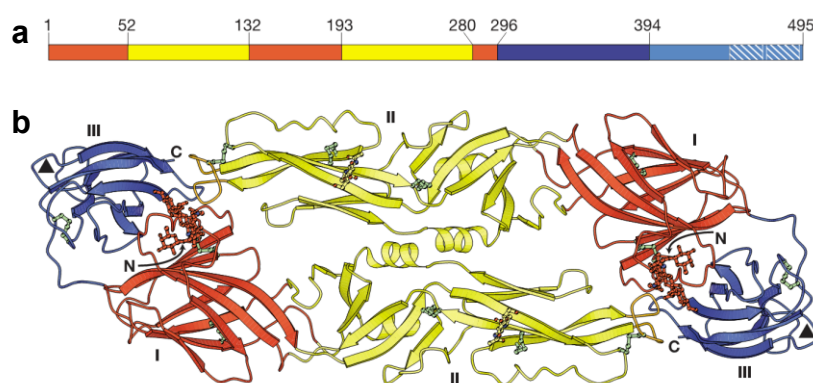


Figure 7. Structure of dengue virus soluble E dimer in mature viruses Showing domain I in red, domain II in yellow, domain III in blue. **a)** Representation of Dengue virus E primary structure including the 100 residue C-terminal section missing from the soluble form that includes the stem segments (solid cyan) and the transmembrane anchors (stripped cyan). **b)** Upper view of the soluble E dimer from Dengue virus 3 (Reprint from reference 85).

Domain I (DI) occupies a central position in the E monomer⁸⁶. As pictured in Fig. 7a, this linearly discontinuous structure it is formed by approximately 120 aminoacids distributed in three different segments of the protein that, when folded together, form an 8-stranded β -barrel with an axis parallel to the viral membrane that serves as organizing point for the whole protein^{45,85,87}.

Domain II (DII) is formed by two large peptidic sequences intercalated between the segments that form DI. These two loops fold together forming an elongated finger-like

structure with an antiparallel β -sheet of 5 strands and 2 α -helices that form a highly stable core from which an elongated 3-stranded β -sheet expands distally forming two loops^{45,87}; the most distal loop (cd loop) carries a highly hydrophobic glycine-rich sequence, conserved in closely-related flaviviruses, that serves as the internal fusion loop (FL)^{17,45,88}. The flaviviral FL is strongly constrained by several intra-molecular forces that maintain its structure even when exposed to highly polar environments, which makes viral infection possible^{87,89}. Additionally, DII provides the surface where the main interactions necessary for stabilizing the dimer conformation occur⁴⁵.

Domain III (DIII) is the C-terminal domain, and the only linearly continuous domain⁹⁰. Structurally, it's formed by an immunoglobulin constant region-like β -barrel in which the sides form an internal hydrophobic pocket (together with DI) and the outer-lateral, most outward-prominent surface of the E dimer^{45,86}. Regarding its functions, DIII is thought to contain the putative receptor-binding sites responsible for interacting with the host cell initiating the infection process⁴⁰. This is supported by reports in which highly neutralizing antibodies have been mapped mainly to DIII epitopes^{17,91,92} and soluble recombinant version of DIII are able to block infection⁹³. Besides this function, DIII has also been implicated in determination of host range, tropism and virulence^{17,45}.

In addition to these well-defined domains, the E ectodomain has two extra functional regions that fulfil very important roles: the hinge region formed by the 4 peptide strands that connect DI and DII fragments, and the linker that connects DI and DIII.

The four peptide strands that form the hinge region are loosely packed in the interface between DI and DII, and provide the required flexibility for pH-induced conformational changes in the orientation of DII that are necessary at several stages of the viral lifecycle⁸². The presence of conserved glycines and the distribution of polar and non-polar aminoacids facilitate this pivot-type motion^{45,94}. In fact, the rotation of DII about the hinge region is essential for viral infectivity, tropism and maturation^{94,95}.

The DI-DIII linker is a short (11 residues), moderately-conserved, poorly-organized peptide. Recently, de Wispelaere and Yang conducted a study in which they tried to pin down the functions of this linker by mutating aminoacids conserved in all four dengue virus serotypes⁹⁶. Their findings, along with other crystallographic studies, suggest an important role of this linker in the assembly of the viral particles and in the process of host-cell infection by aiding in the trimerisation of the E protein^{87,89,96}.

The last ≈ 100 aminoacids of E contain the stem and anchor regions that connect the E ectodomain to the viral envelope. The stem region is directly linked to the C-terminal end of DIII and consists of ≈ 50 aminoacids distributed in two consecutive amphipathic α -helices (EH1-EH2), partially embedded in the outer layer of the viral lipid membrane, separated by a highly-conserved linker-like aminoacid sequence (CS)^{44,97,98}. The remaining ≈ 50 residues constitute the anchor region, formed by two transmembrane α -

helix domains (ET1-ET2) that cross the viral lipid bilayer in an antiparallel coiled-coils configuration with an stretch of 6 non-hydrophobic aminoacids between them (Fig. 8)^{44,98}. It has been shown that the stem region plays a big role in viral assembly by neutralizing the electric repulsion between the negatively-charged inner surface of the E ectodomain and the non-polar viral membrane^{99,100}, and in the stabilization of the PrM-E heterodimer^{98,101,102}. The stem region also participates in the viral entry process by aiding in the trimerisation of the E protein that is required to expose the FL^{98,101}. The anchor region, on the other hand, has been related to important functions regarding the assembly and release of mature viral particles^{98,100,103}.

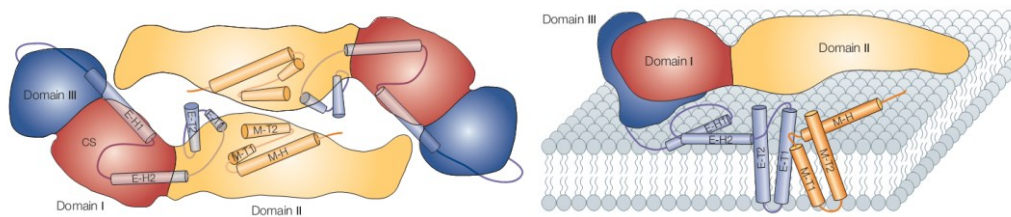


Figure 8. Top view (left) and side view (right) diagrams of the dengue virus E protein ectodomain and transmembrane domains. The stem-anchor region of E and the transmembrane helices of M (M-H, M-T1 and M-T2) proteins are shown in blue and orange, respectively (Reprint from reference 40).

Dengue virus E protein has two conserved N-linked glycosylation sites at asparagine (Asn)67 in DII and Asn153 in DI. The Asn153 site is conserved in flaviviruses while the Asn67 is present exclusively in dengue viruses^{45,85}. Structural studies proved that both glycosylation sites are used in mammalian cells and that each one carries a pentasaccharide mannose 3 core that is common to all N-linked oligosaccharides⁸⁵. Usually, the functions attached to glycosylations on viral proteins include guidance in proper folding of the protein, adequate traffic in the secretory pathway, interaction with cellular receptors and immunogenicity¹⁰⁴. Although there are contradictions about the role of these sites in dengue viruses, available data indicate that they might play an important role in tropism, pathogenesis, assembly of infective particles and cellular attachment^{105,106}. Stronger evidence supports the notion that both sites are associated with increased infectivity and proper release of mature viral particles¹⁰⁷. The dengue-specific glycosylation site at Asn67 has been implicated in mediating the attachment of the virus to the C-type lectin DC-SIGN expressed on dendritic cells and a subset of macrophages in the skin, which are thought to be the first target cells during dengue virus infection^{105,108}.

The E protein is a highly dynamic structure that undergoes severe conformational changes during the viral cycle. Each one of these intermediates (immature, pre-fusion and post-fusion trimeric conformations) is related to a particular purpose during a stage of the viral lifecycle and will be discussed later. The typical antiparallel dimeric conformation of the mature E protein is stabilized by a mixture of hydrophilic and hydrophobic interactions that take place along DII but that are concentrated in two regions: the inner face of the

opposing DIIs concentrate most polar interactions between the hydrophilic side chain of the α -helices of each monomer, while the hydrophobic interactions occurred predominantly at the distal end of DII where the fusion loop is buried within a hydrophobic pocket formed at the interface of DI and DIII of the opposed monomer^{45,84,85,87-89,94,99}. This last interaction is further promoted by the presence of the glycosylation at DI that further stabilizes the dimer by keeping DII in place^{45,89}.

2.5.2. Non-structural (NS) proteins

2.5.2.1. *Non-Structural Protein 1*

The NS1 protein is a variably glycosylated, ≈ 46 kDa, protein that is translocated into the ER during translation of the viral mRNA^{17,21}. NS1 folds into its mature structure and forms highly stable dimers with affinity for membrane interaction^{109,110}.

NS1 is mainly intracellular, but can also follow the secretory pathway and can localize to the cell membrane or be secreted^{17,110}.

Although its exact involvement remains unclear, studies have shown that intracellular NS1 plays an essential role as a cofactor for viral RNA replication^{111,112}. The finding of NS1 co-localising at sites of RNA replication and with dsRNA intermediates, have suggested a structural role within the replication complex in close interaction with NS4A^{111,113,114}.

Extracellular NS1 can be found as a secreted hexamer or as a membrane-bound dimer^{115,116}. The secreted soluble forms have been implicated in viral pathogenesis¹¹⁷⁻¹¹⁹, evasion of immune response^{120,121} and induction of autoimmunity^{122,123}, while the membrane-anchored NS1 has been linked with a signal transduction capacity that might affect viral replication inside infected cells¹²⁴.

More recently, a series of studies have successfully connected NS1 from dengue virus to disruption of the endothelial cell integrity by inducing degradation of proteoglycans present on the cell surface¹²⁵ and by inducing a strong pro-inflammatory response via TLR4 signalling, leading to endothelium hyper-permeability^{126,127}.

2.5.2.2. *Non-Structural Protein 2A*

NS2A is a ≈ 22 kDa, hydrophobic protein (Fig. 5) that is not well conserved among flaviviruses but seems to share similar structural features, the most important being the presence of an internal hydrophobic domain that suggests a transmembrane topology^{17,128}. Regarding its functions, several studies have demonstrated a role for NS2A in viral assembly^{129,130}, particularly in the process of generating viral-induced membrane structures which seem to be pivotal for the assembling process¹³⁰. Additionally, NS2A not only co-localises with the double-stranded RNA (a replication intermediate) foci within infected cells, but also binds strongly to the 3'UTR region of the flaviviral genome, NS3

and NS5¹³¹. Taken together these results suggest that NS2A might also have a role in RNA replication with a function in coordinating the transition between RNA replication and viral assembly that leads to the generation of new viral particles^{86,131}.

Recently, NS2A has also been linked to a role in modulation of the host immune response by inhibiting interferon signals in infected cells^{132,133}.

2.5.2.3. Non-Structural Protein 2B

NS2B is a (≈14kDa) membrane-associated protein that associates with NS3 to activate the serine protease activity of NS3. In this sense NS2B serves as a cofactor for the viral protease^{134,135}. Structurally, NS2B is formed by a hydrophilic central domain between two hydrophobic regions at the N- and C-terminus¹⁷. Residues 67-80 within the hydrophilic domain are critical for the cofactor activity of NS2B and had been shown to change the structure of NS3 activating its proteolytic activity^{136,137}. The hydrophobic domains of NS2B also mediate the association of the NS2B-NS3 complex to the ER membrane^{138,139}.

2.5.2.4. Non-Structural Protein 3

NS3 (≈70kDa) is a multifunctional protein that contains several enzymatic activities involved in the processing of viral proteins and replication of viral genome^{86,140}.

The N-terminal third of NS3 bears the chymotrypsin-like, serine protease catalytic domain involved in post-translational processing of the viral polyprotein^{17,134,141}. The enzyme is formed by two β-barrels (each one consisting of 6 β-strands) with the catalytic triad (His51, Asp75, Ser135) located between them^{86,142}; binding of NS2B stabilises NS3 structure activating the substrate binding site and the protease activity^{136,143}. The NS3 cleavage site requires two basic aminoacids (dibasic (Arg/Lys)-Arg motif) followed by a small, un-branched aminoacid at P1' position¹⁴⁴.

Following a poorly conserved inter-domain linker, the C-terminal portion of NS3 carries domains with several functions associated with RNA replication including: sites for RNA binding, RNA helicase, RNA-stimulated nucleoside triphosphatase (NTPase) and RNA triphosphatase (RTPase)^{17,140}. The helicase structure comprises 3 subdomains: the helicase activity is contained within a complex of β-sheets and α-helices and has been proven to be essential for viral replication^{142,145}; the subdomain III is unique to the flaviviruses and contains a small region of highly basic aminoacids that may serve as site for protein and RNA binding, specifically to the 3'SL and the NS5 protein, during viral replication^{146,147}. In addition to these proposed functions, subdomain III also encodes for the RTPase activity involved in dephosphorylating the 5' end of the genomic RNA for the subsequent addition of the cap^{148,149}.

NS3 has also been involved in other functions regarding virus assembly¹⁵⁰ and modulation of intracellular environment¹⁵¹⁻¹⁵³. In particular, the NS2B/NS3 protease complex is directly involved in cleaving the human mediator of interferon regulatory factor 3 activator (STING) which down-regulates the intracellular defence against viral infections¹⁵⁴

2.5.2.5. Non-Structural Protein 4A

NS4A is a small, hydrophobic protein (≈16kDa) that consists of an initial cytoplasmic N-terminal sequence, a central hydrophobic region formed by three different domains that are tightly associated with the ER membrane, and a final C-terminal sequence that acts as a signal for the translocation of NS4B into the ER^{155,156}. Among its many proposed functions, NS4A is reported to be a cofactor for the NS3 helicase¹⁵⁷⁻¹⁵⁹; additionally, the membrane-interacting domains of the protein have been reported to participate in inducing membrane rearrangements for the viral replication site as well as in anchoring the replication complex^{157,160-162}.

Additional functions seem to involve modulation of the cellular environment¹⁶³ and inhibition of immune response by blocking IFN signalling^{133,164}.

2.5.2.6. Non-Structural Protein 4B

NS4B is a small (27kDa) hydrophobic protein closely associated with the ER membrane through at least 4 transmembrane domains, although its complete structure and topology is not fully understood^{156,165,166}. Little is known about the actual role of NS4B during viral infection but it has been proved to be essential for viral RNA replication^{167,168}. In particular, the protein co-localises to the RNA replication sites¹⁶⁹, aids in the disassociation of the NS3 and the replication intermediates^{168,170}, and induces the formation of specialized membrane compartments for replication¹⁶⁸.

In addition to these functions, NS4B has also been described as suppressor of cellular interference RNA and immune responses¹⁷¹⁻¹⁷³.

2.5.2.7. Non-Structural Protein 5

Located at the C-terminus of the viral polyprotein, NS5 is the largest (≈105kDa) and most conserved of the flaviviral proteins with two main functions: it serves as the viral RdRp and as a methyltransferase (MTase) involved in cap-processing of the RNA^{17,174}.

The N-terminal region of NS5 carries the MTase function with close homology to S-adenosyl-methionine (SAM) dependent methyltransferases motifs^{175,176}. The enzymatic domain is conserved, both in structure and function, among all flaviviruses, with data showing its capacity to sequentially catalyse a guanine N-7 and a ribose2'-O-

methylation^{174,177}. Its involvement in capping of the viral genome was further supported by studies demonstrating the lethal effect of mutations disrupting the MTase activity^{178,179}.

The C-terminus of NS5 contains the viral RdRp enzyme, which has significant homology to other single-strand, positive-sense RNA viruses' polymerases^{180,181}.

The central region between the MTase and the RdRp domains has nuclear localization signals that effectively guide a fraction of the viral protein to the nucleus of infected cells suggesting additional roles for NS5 probably involving suppression of cellular immune responses although this has not yet been confirmed^{182,183}. Finally, this interdomain region also mediates the interactions with the 3'SL element of the viral genome¹⁴⁶ and with NS3, which regulates its NTPase-RTPase activities¹⁸⁴.

Moreover, in the case of dengue, NS5 mediates STAT2 degradation by interaction with the host UBR-4 (ubiquitin protein ligase E3 component N-recognin 4) protein, thus interfering with the IFN signalling pathway¹⁸⁵.

2.6. Viral Replication Cycle

Although the understanding of the dengue virus replication cycle is far from complete, there's enough evidence to believe that it shares many characteristics with other members of the Flaviviridae family^{17,186}. The general process is shown in Fig. 9 and the most relevant stages are described in this section.

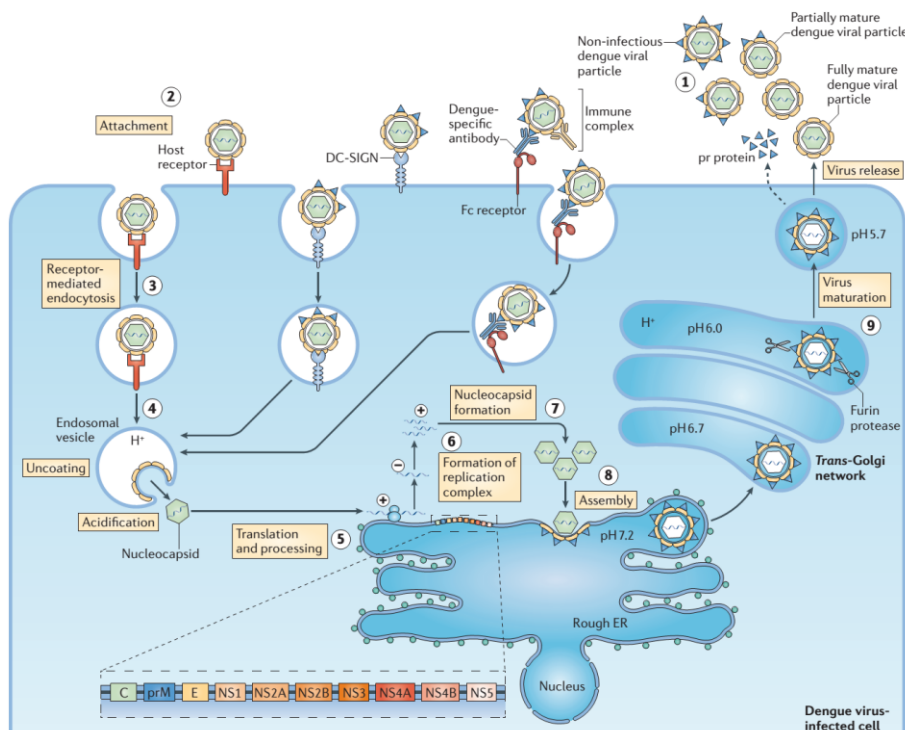


Figure 9. Schematic representation of the general steps involved in the dengue life cycle. 1) Depending on the level of PrM cleavage, dengue viral particles exist as a mixture of different structures, fully mature particles are infectious while fully immature particles aren't; depending on the amount of PrM uncleaved, some partially mature particles can be infectious. The virus attaches to the host cell receptor (or the Fc γ -receptor in the case of ADE) (2) and enters the cell via clathrin-mediated endocytosis (3). Acidification of the early

endosome triggers viral fusion with the endosomal membranes and the liberation of the nucleocapsid (4). The viral ARN is then translated and processed to produce viral proteins (5) before commencing the replication process (6). The new viral genomes are packaged by the capsid protein (7). Viral assembly initiates when the nucleocapsid buds into the ER (8). The immature viruses follow the secretory pathway and arrive to the trans-Golgi network where low pH triggers maturation thru PrM cleavage by furin protease (9). Pr remains attached until it's released by the neutral pH of the extracellular environment (Reprint from reference 186).

2.6.1. Attachment-Entry-Fusion:

In this initial step, the virus attaches to the membrane of the target cell and interacts with the cellular receptor inducing its internalization via clathrin-mediated endocytosis¹⁸⁷.

Although there is compelling evidence indicating that the viral cell receptor-binding motif resides somewhere within DIII, the identity of the cell receptor(s) is still unknown¹⁸⁸. Several groups have identified cell receptors that are able to bind dengue virus' E protein, including: glycosaminoglycans (heparan sulphate in particular)¹⁸⁹, stress response proteins¹⁹⁰, heat shock proteins¹⁹¹, mannose receptor in macrophages¹⁹² and the already mentioned DC-SIGN and L-SIGN^{105,193}. The general consensus is that dengue virus entry into the host cell probably involves a receptor complex composed of several molecules in which the glycan-binding receptors serve as an initial interaction to allow close range contacts between the virus and the cell^{188,194}.

Following intake, the exposure to acidic pH inside the endosome triggers E protein trimerisation (Fig. 10) which leads to the exposure of the fusion loop, a necessary step to initiate the fusion process^{17,42}.

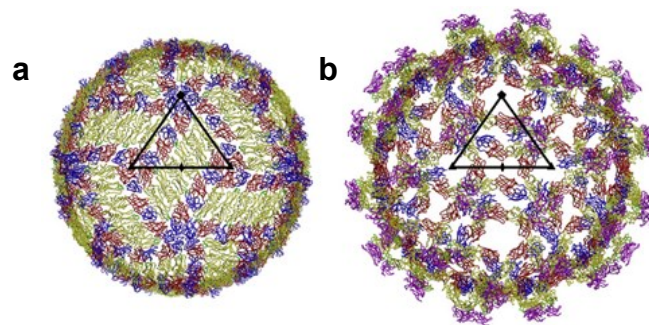


Figure 10. Changes on the viral surface arrangements upon exposure to acidic pH inside the early endosome. Schematic representation of the surface of a mature flaviviral particle at a) neutral pH and b) acidic pH (Reprint from reference 42).

Upon exposure to low pH condition in the early endosome, several key histidine residues along the E protein change their protonation state and become doubly-positively charged, which generates a motion that breaks all the intersubunit contacts on the viral surface thus giving the initial energy required for trimerisation^{84,99,195}. As pictured in Fig. 11, the trimeric structure of E involves a conformational change that reorients the molecule from a horizontal antiparallel dimer into a vertical parallel trimer, where the fusion loops are exposed at the tip of the trimer but retaining their original conformation,

forming an “aromatic ring” that serves as anchor for the fusion process with neighbouring hydrophilic residues and restricting the insertion of the complex to the inner leaflet of the endosome membrane^{87,196}. After anchoring to the endosomal membrane, the E pre-fusion intermediate folds back on itself in such a way that the anchor region is pushed towards the fusion loop into what is known as the post-fusion intermediate. This movement fuses the viral membrane and the endosomal membrane and allows the release of the viral nucleocapsid into the cytosol^{84,87}.

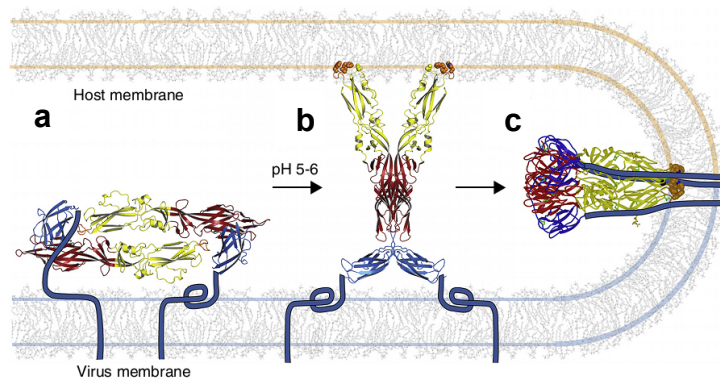


Figure 11. Membrane fusion by flaviviruses. Schematic representation of the conformational intermediates of the E protein during the fusion process. a) Dimeric conformation of the E protein at neutral pH, **b)** Prefusion intermediate conformation of E as the result of the acidification in the early endosome that leads to a rearrangement of the dimer and the exposure of the fusion loops forming an anchor that inserts into the endosome membrane, **c)** Following the insertion into the host membrane the trimeric E suffers further rearrangements that cause a folding back motion which brings the anchor regions and the fusion loop together into a postfusion intermediate that connects viral and host membranes completing the fusion process (Reprint from reference 84).

2.6.2. Translation and polyprotein processing:

After viral entry and genome uncoating, the RNA genome serves as a messenger RNA (mRNA) and is translated by the cell machinery to produce a large polyprotein that is cleaved by host and viral proteases to yield the mature structural and non-structural proteins^{70,86}. The general topology of the viral polyprotein is shown in Fig. 5.

The main mechanism for translation is the typical cap-dependent initiation in which the type 1 cap at the 5' end of the viral genome is recognized by eIF4E that in turn recruits the whole translation machinery¹⁹⁷. In cellular mRNAs, translation is enhanced by the presence of the poly(A) tail and the 3' end of the RNA; in dengue, several secondary structures at the 3' UTR region of the viral genome increase viral translation which seems to be even more important to compete for the recruitment of the translation complex as dengue virus is unable to shut down cellular translation during infection^{197,198}. Additional ways to overcome this translational competition include the generation of viral-induced ER-derived membrane structures where the viral replication, translation and assembly machinery are concentrated¹⁹⁹, as well as a non-canonical, cap-independent alternative

mechanism of translation that ensures viral translation in adverse cellular conditions like high osmolality or reduced levels of eIF4G²⁰⁰.

As represented in Fig. 5, the viral polyprotein is co-translationally cleaved by the host signal peptidase at sites between C-PrM, PrM-E, E-NS1 and at the C-terminus of NS4A while the viral protease (NS3/NS2B) is responsible for the cleavages at NS2A-NS2B, NS2B-NS3, NS3-NS4A, NS4A-NS4B and NS4B-NS5⁸⁶. The enzyme that cleaves at the NS1-NS2A site is unknown¹⁷. Cleavage of Pr from M is carried out by the trans-Golgi localised host enzyme furin.

2.6.3. RNA Replication:

Flavivirus replication occurs within viral-induced membrane structures, which facilitate RNA replication by increasing the local concentration of the necessary components and also by providing a scaffold for the assembly of the replication complex^{86,201}. In addition, these membrane-sites may fulfil a role in hiding the viral intermediates from the cellular defences²⁰².

Available data show that the flavivirus replication complex probably consists of a double-stranded RNA template in association with all the non-structural viral proteins and some host factors within the aforementioned membrane structures (a proposed model is shown in Fig. 12)^{17,203,204}.

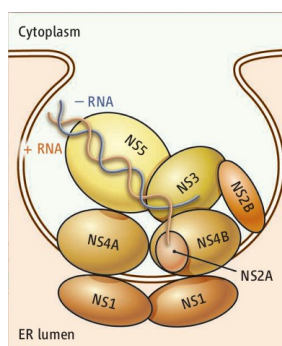


Figure 12. Schematic representation of the dengue virus replication complex. Derived from available literature in flavivirus replication, this hypothetical model shows the replication complex (RC) anchored within viral-derived membrane vesicles facing the cytoplasm and has been variably shown to comprise a double-stranded RNA template, the viral non-structural proteins and host derived factors (Reprint from reference 204).

Viral replication begins with the synthesis of a complementary minus strand to the genome RNA that serves as a template to generate additional genomic RNA molecules, and forms the double-stranded intermediated (named replicative form (RF))¹⁷. Dengue virus RNA replication is a semiconservative, asymmetric process in which the positive-sense RNA is synthesized between 10 to 100 times more than the negative strand²⁰⁵; additionally, the process is coupled with the RTPase activity of NS3 and the MTase activity of NS5 to produce the 5' cap of the viral genome, thus the newly-made positive-

sense RNA genomic molecules can be further used for replication or translation or packaged into new virion^{70,86}.

2.6.4. Assembly, Maturation and Release of Viral particles:

Although it remains a highly unknown process, viral assembly occurs within the ER-derived membrane structure induced by the virus in the infected cells²⁰⁶. Viral assembly begins when the capsid protein, attached to the cytosolic side of ER membrane, associates with one molecule of the RNA viral genome and forms the nucleocapsid⁷⁰.

Once formed, the nucleocapsid acquires the viral envelope by budding into the ER lumen where the PrM and E proteins remain attached by means of their transmembrane anchors⁷⁰. The budding particles enter the ER lumen as immature non-infective particles with a characteristic “spiky” appearance due to the presence of the covalently-linked Pr-M peptides that form heterodimers with the E protein across the viral surface^{17,80}. Maturation occurs as the viral particles are transported through the secretory pathway where the viral surface undergoes major changes due to the acidic environment of the trans-Golgi network (TGN)^{44,207}. As shown in Fig. 13, exposure to a low pH induces significant rearrangements of the surface proteins that shift from an E trimeric spike-like conformation to an E dimeric antiparallel herringbone organization that exposes the furin cleavage site on PrM²⁰⁷. The cleavage of the Pr peptide renders mature infective viral particles. However, the Pr peptide remains attached to the E dimer acting as a cap-like structure to protect the fusion loop from premature exposure that could lead to viral-membrane interaction with membranes of the secretory pathway before viral release^{78,80}. After mature particles are secreted from the cell, the change to a neutral pH environment stabilises the E dimeric formation and disassociation of the Pr peptide⁶⁷.

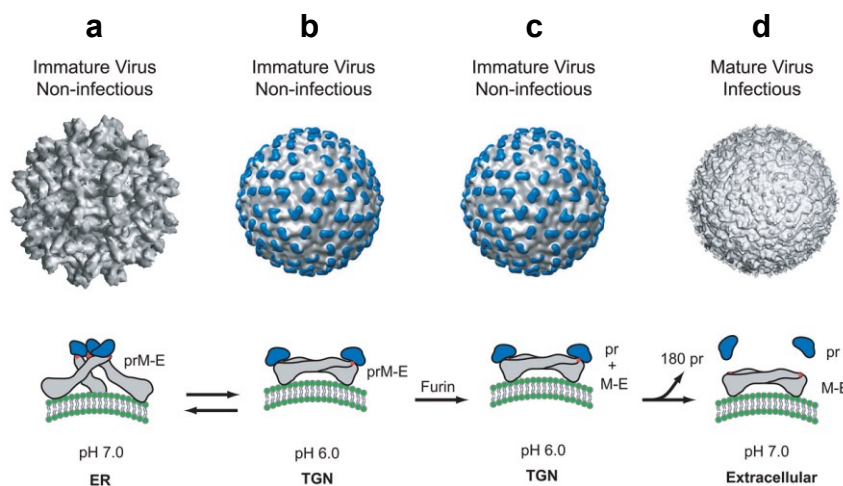


Figure 13. Structure of the dengue virion and conformation of the E protein during maturation. a) Cryo-EM reconstruction of the immature virion at neutral pH after budding into the ER lumen, where the E exist as a Pr-E heterodimer in a trimeric conformation, hence the “spiky” appearance of the virus. b) Structural rearrangements of the immature particle at the low-pH in the TGN where the Pr-E heterodimer change to a flat

dimeric conformation. **c)** The structural change exposes the furin cleavage site in PrM that remains attach to the E protein after cleavage. **d)** The mature viral particle is secreted from the cell into a neutral pH environment where the Pr peptide is released (Reprint from reference 67).

In practice, however, the process of maturation appears to be inefficient and many particles, usually referred as mosaic as they contain both mature and immature regions, are released with various degrees of Pr cleavage, the extent of which determines whether they are infective or not^{207,208}. If Pr is not cleaved the E protein rearranges it's dimeric organisation at neutral pH and reverts to the “spiky” intracellular configuration causing a shift on the viral surface with the formation of 60 prominent and irregular Pr-E trimeric spikes that increase the particle diameter by about 100Å (Fig. 14)²⁰⁷. Previous studies have demonstrated that fully and partly immature particles constitute approximately 40% of all particles released from infected cells²⁰⁷. In addition subviral particles (also known as virus-like particles (VLPs)) are routinely observed in flaviviral infections. These are small (≈ 300 Å), smooth, mature-like particles formed by the E and M proteins and the lipid bilayer without the nucleocapsid²⁰⁹. These particles undergo the same maturation process as the mature virions, and can be artificially produced from cells by expressing the genes coding for the PrM-E proteins⁴⁰. As a consequence of all these viral and subviral forms released from infected cells, and together with viral “breathing” and the temperature-dependent changes on the configuration of the viral surface, the level of structural variability in flaviviruses is very large, and has significant implications not only for the infection process but also for the immune response against it.

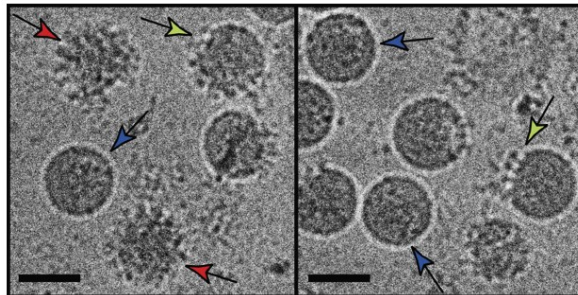


Figure 14. Cryo-EM images of two WNV preparations. Red arrows indicate fully immature “spiky” particles while blue arrows indicate fully mature “smooth” virions. Partially mature mosaic particles are indicated by green arrows. Bar represents 500 Å (Reprint from reference 210).

3. DENGUE DISEASE

3.1. Virus-Vector Interactions

Two different transmission cycles have been proven to exist for dengue virus: the sylvatic cycle and the endemic urban cycle (Fig. 15)⁷. The urban endemic cycle is the most important for dengue and humans serve both as reservoir and amplification host. This cycle is dependent on *A. aegypti* and *A. albopictus* as transmission vectors^{53,211}.

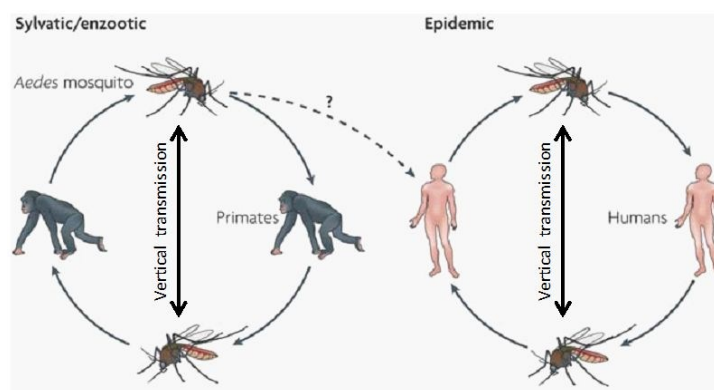


Figure 15. Dengue transmission. Dengue virus has been shown to be maintained through two different transmission cycles: the sylvatic cycle which involve mainly non-human primates as host and forest canopy-dwelling mosquitos as vectors and the epidemic urban cycle in which the virus is transmitted among humans by *Aedes spp.* mosquitos, principally *A. aegypti* and *A. albopictus* (Reprint from reference 212).

The efficiency of the endemic cycle is favoured by the ecology and behaviour of *A. aegypti*, the most important vector in this scenario: it is closely associated with human dwellings and has adapted to urban environments; vector competence is further increased by the fact that females feed almost exclusively on human blood during the gonotrophic cycle. In addition, unlike many other mosquitos, *A. aegypti* is active during daylight hours, and feed on several hosts during each blood meal, which further increases the risk of transmission^{53,213}.

A. albopictus serves as a secondary vector of dengue and is found mainly in forest areas. Both species show high water requirements for breeding and lay their eggs in water-holding vessels or tree holes, which implies a peak of mosquito densities, and disease incidence during rainy seasons^{211,214}.

In the sylvatic cycle dengue virus circulates among non-human primates that serve as hosts and reservoirs. Some of the mosquitos species involved in this transmission are *A. africanus*, *A. leuteocephalus* and *A. furcifer* among others^{7,214}.

Once ingested, the virus infects the epithelial cells of the midgut where it spreads into the haemocoel, finally infecting the salivary glands from which the virus is transmitted to the vertebrate host during the blood meal²¹⁵.

3.2. Virus-Host Interactions

In the case of dengue, identification of the target cells and spread of the infection in vertebrate hosts has proven to be difficult. However, available data support the notion that cells from the reticuloendothelial system (consisting primarily of monocytes, macrophages and derived cells) are the main target of the viral infection with the Langerhans immature dendritic cells in the skin serving as the initial replication target for the virus^{108,216}. These cells migrate into lymph nodes where the virus spreads to other cells from the macrophage-monocyte lineage⁵³. The infection is amplified in lymph nodes and then

spreads through the blood via infected monocytes to the spleen, liver and bone marrow that serve as additional sites for amplification²¹⁷.

Data demonstrating viral dissemination in solid organs has been inconsistent, with studies reporting detection of viral antigens in lymphocytes, hepatocytes, cardiac fibres and pneumocytes^{218,219}; however, any organ containing reticuloendothelial cells could eventually serve as a site for viral replication²²⁰. Although relevant during the pathogenesis of infection¹²⁵, the role of endothelial cells within the infection is not yet confirmed because they are susceptible to viral infection *in vitro* but infection of the endothelium does not seem to be present *in vivo* even in severe manifestations of the disease⁵³.

3.3. Clinical Manifestations of the disease

Dengue infection causes a systemic disease in humans with a wide symptomatic spectrum that ranges from non-symptomatic to severe haemorrhagic disease²¹¹.

The majority of dengue infections, especially those involving children and people in highly endemic regions, are asymptomatic²²¹. In patients with clinical disease, the illness can be divided in three different phases: febrile, critical and recovery; the main aspects of each phase are resumed in Fig. 16. Additionally, depending on the clinical evolution of the patient, the infection can be classified in three different conditions: the non-severe form of the disease called Dengue Fever (DF), and the more compromising manifestations named Dengue Haemorrhagic Fever (DHF), and Dengue Shock Syndrome (DSS)^{211,222}.

Paradoxically, the clinical management of the patient is simple and effective²¹¹. The key resides in a deep knowledge and early recognition of the disease to initiate correct and timely treatment²²². However, given the relatively common symptoms involved in the initial stages, early diagnosis of the disease is complicated by the vast differential diagnosis²¹¹.

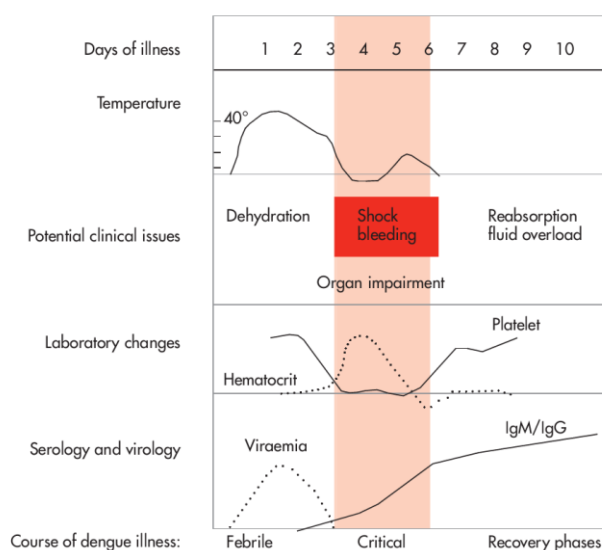


Figure 16. The course of dengue illness (Reprint from reference 211).

3.3.1. Phases of the Dengue disease

3.3.1.1. The Febrile Phase

After an incubation period of 4-7 days patients develop high fever (39-41.5°C) that marks the beginning of the febrile phase. This phase usually last between 2 and 7 days and is accompanied by generalised body ache, arthralgia, myalgia, frontal headache, retroocular pain and skin maculopapular rash (starting in the trunk and expanding to the face and extremities but not affecting palms or soles); together, these findings explain why dengue disease is also known as “*Breakbone fever*” in many countries²¹¹. Although not as common, some haemorrhagic manifestations like mucosal bleeding (mainly on the nose and gums) can be seen⁵. Significant laboratory findings include neutropenia with associated lymphocytosis (including a high percentage of atypical lymphocytes) and thrombocytopenia⁵.

At this stage of the disease, severe and non-severe forms of the disease are indistinguishable and monitoring the patient is crucial to avoid complications that could lead to a fatal outcome²¹¹.

3.3.1.2. Critical Phase

The febrile phase finishes when the patient's temperature drops back to normal levels, or slightly higher (37.5-38°C). This event may be accompanied by an increased in capillary permeability which leads to a significant plasma leakage and marks the start of the critical phase²¹¹. Capillary permeability also marks the difference between the non-severe and severe forms of the disease: patients without an increase in capillary permeability will recover without complications marking the end of the typical course for DF, while those suffering from capillary leakage (DHF) may show an increasingly worsening condition as a result of the reducing plasma volume that may end in a very complicated scenario of severe shock (DSS)^{5,211,222}.

Significant extravasation of plasma through the gaps between endothelial cells last between 24-48 hours. Patients suffering from DHF usually present haemorrhagic features like scattered petechiae, purpuric lesions, epistaxis and mucosal bleeding; in addition, patients may present ascites and pleural effusion^{211,221}. Blood analysis during this phase shows haemoconcentration as evidence of the vascular leak of plasma, together with a more severe thrombocytopenia and absolute granulocytopenia^{5,17}.

If plasma leakage is not treated, patient develops severe hypovolemia that results in circulatory failure and shock (DSS)⁵. Some warning signs of DHF progressing into DSS are: intense abdominal pain, persistent vomiting and a sudden change from fever to hypothermia with profuse sweating²²¹. At this point treatment is critical as prolonged shock and the consequent hypoxia could develop into multiorgan failure compromising the life of the patient²¹¹.

3.3.1.3. *Recovery Phase*

If the patient survives the critical phase, the body reabsorbs the leaked fluid thus returning to a stable haemodynamic state²¹¹. However, prolonged asthenia, mental depression and cardiac abnormalities are also common in infected patients after the fever breaks²²².

In addition, rare manifestations like encephalopathy, liver failure or cardiomyopathy, have been described in several patients during the course of a dengue infection²¹¹.

3.4. **Epidemiology**

In order to get a clear grasp of dengue virus' impact on the different aspects of human life, it is fundamental to understand the viral geographic distribution and the overall burden of the disease in endemic countries²²³. This is also critical to determine the best way to properly use and distribute the limited resources available for disease control²¹¹. In addition, epidemiological data is crucial when performing clinical surveillance and for scoping preventive measures when facing epidemics or outbreaks in endemic countries^{6,224}.

Together with the current situation of Zika virus²²⁵, dengue is the most rapidly spreading mosquito-borne viral disease in the planet and a major health concern for nearly half of the world's population, and is also one of the principal causes of paediatric hospitalization and deaths in endemic countries^{211,226}. Recent data indicates that up to 3.6 billion people are currently living in areas under primary risk of infection (Fig. 17)^{6,26}. Although estimates vary, there are over 300 million infections each year with close to 100 million symptomatic cases and over 500,000 episodes of DHF/DSS, with an annual mortality reaching over 20,000 deaths^{224,226,227}. Moreover, due to poor and passive surveillance systems in endemic countries, difficulties in the diagnosis and low case fatality, the true incidence and impact of dengue is probably much higher^{6,223}; in fact, Samir Bhatt and others developed an algorithm to create an evidence based map of dengue risk and estimates of worldwide infections on the basis of the global population in 2010, obtaining total infection figures that were more than three times those predicted by the World Health Organization (WHO)²²³.

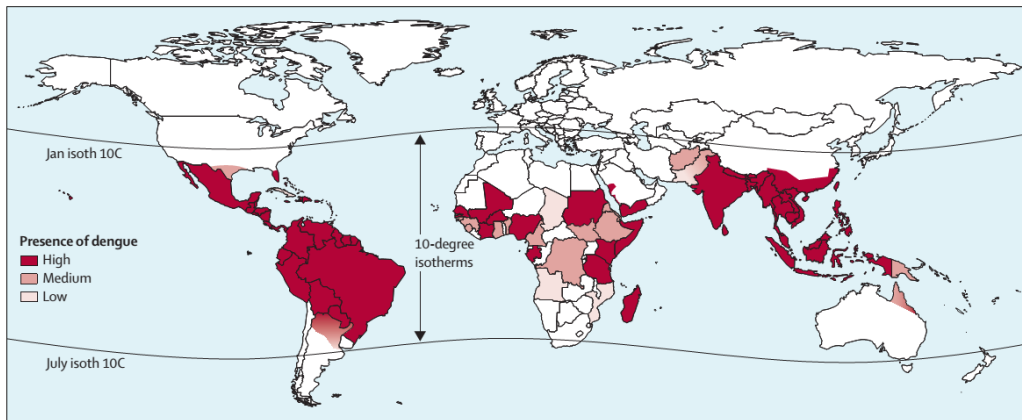


Figure 17. The global distribution of dengue, 2014 (Reprint from reference 26).

The remarkable spread of this infection means that nearly 120 countries are endemic for dengue with the incidence of the disease showing a 30-fold increase over the past 5 decades (Fig. 18), which is probably among the main reasons why the WHO classified it as the “most important mosquito-borne viral disease in the world”²²⁴.

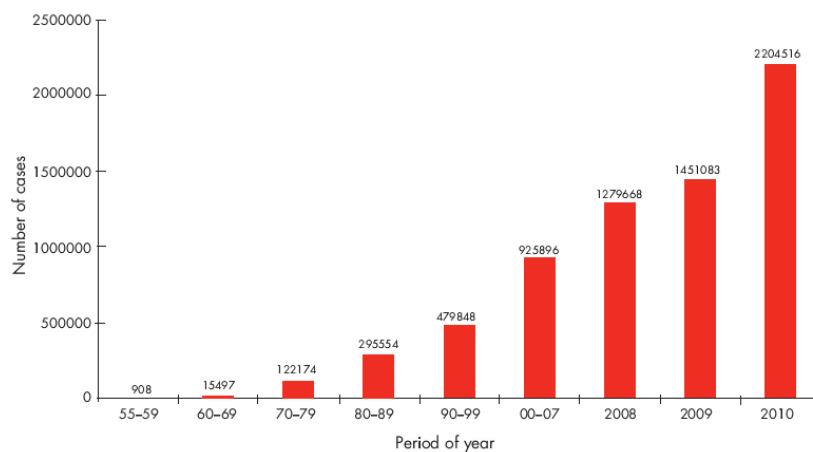


Figure 18. Average number of cases reported to the WHO from 1955-2007 and number of cases reported in recent years (2008-2010) (Reprint from reference 224).

Dengue epidemics are a significant health, economic, social and even political burden on the countries where they occur, generating consequences that are difficult to measure¹¹. Indeed, determine the actual economic impact (cost) of dengue in endemic countries has proven to be a very difficult task^{11,228}. A recent study made by Shepard and collaborators²²⁹ concluded that dengue epidemics in America cost the region around 2.1 billion US dollars per year on average, without including the cost of vector control measures. Similar studies have been done generating figures that in general underestimate the real cost of the disease^{25,230,231}.

3.4.1. Distribution of the disease

Despite the uncertainty in the actual number on infections, there's enough evidence showing dengue transmission in every populated continent.

Asia: Although dengue is a global concern, 75% of the population exposed to the infection live in the Asia-Pacific region. Within these regions, Southeast Asia is by far the most affected with 1.3 billion people at risk and rates for severe dengue disease almost 20 times higher than those reported in America^{211,230}. In the last decade, the disease expanded to almost every country in the region²³¹.

Africa: Little is known about the current situation of dengue in Africa mainly because the disease is not officially reported to the WHO and there's a higher probability of under-recognizing the disease amidst the wide variety of similar infectious conditions that affect the region, especially malaria^{6,211}. However, there's evidence suggesting that the number of infections and the spread of the disease are increasing with endemic dengue transmission in 34 countries and confirmed isolation of all four serotypes in the continent^{211,232}.

America: Almost all American countries are hyperendemic with active dengue transmission²²⁴. The situation is more severe in Latin America where the four serotype are in circulation and the vast majority of the infection events are reported: 65% of them in the region comprising Brazil, Paraguay and Argentina followed by the Andean region of Colombia, Ecuador, Peru, Venezuela and Bolivia with 20% and Central America and the Caribbean reporting almost all remaining cases^{6,211}. In North America, almost all the reported dengue infections are imported from endemic regions although outbreaks in Hawaii and Texas have been reported^{211,233}.

Europe: After the last documented epidemic of dengue in Greece between 1926-1928, no transmission has been reported in the region and data available indicates that most of the reported cases are imported²¹¹.

3.4.2. Factors influencing incidence and geographic spread of DENV

As with any vector-borne infectious disease, dengue's epidemiology is determined by an epidemiological triad that includes the human host, the pathogen and the mosquito vectors^{11,226}. Although there are many factors that have positively influenced the incidence and spread of the disease, there are some that are considered among the principal drivers for dengue's current epidemiological landscape:

3.4.2.1. *Lack of efficient mosquito control*

Prevention and control of dengue disease currently depends on effective control of the mosquito vector⁵; however, the scarce resources available have been mostly used for emergency spraying (Ultra low volume) of organophosphate insecticides in response to reported cases of dengue with the objective of interrupting transmission¹¹. This control method has higher impact in society because of its visibility, but fails in controlling the spread of the disease because it is meant to interrupt transmission by killing the infected

adult females that usually dwell indoors where the treatments don't reach and have no effect on larvae that remain behind to restart the transmission cycle^{1,11,15}. The only effective way to control the vector is to pair the insecticide spraying with elimination of breeding sites¹.

3.4.2.2. Increase in travel and trade due to globalization

The contributions of increased mobility in vector and host populations may be one of the top reasons behind the recent increase in transmission and spread of dengue. The globalized economy has created a global reality of international travel and exchange of goods in which the movement of infected people via air travel has been a relevant driver in the expansion of the disease^{6,11}. International trade, especially that involving sea shipment, can also be involved in the introduction of vectors in new regions⁶.

3.4.2.3. Urbanization-related factors

Unplanned population growth paired with inadequate housing conditions especially regarding water supply, sewage or waste management have facilitated the creation of urban breeding sites for the mosquito vector, increasing vector densities in intimate association with crowded human populations resulting in a higher risk of dengue transmission^{5,6}. Indeed, data from seroprevalence studies performed between neighbouring border cities in Northern Mexico and Southern Texas, indicate that socioeconomic factors have a significant impact over dengue's dynamics, concluding that more favourable conditions correlated with reduced larvae breeding sites and lower dengue transmission^{233,234}.

3.4.2.4. Climate factors

Climatologic conditions play a fundamental role in mosquito biology, especially in the transmission cycles of the virus they transmit, with higher temperatures increasing not only the distribution of the vector, but also the rate of larval development and the biting rate of a complete blood-meal while reducing the replication time of the virus in the vector, thus facilitating the overall transmission of the pathogen²³⁵. Several studies have shown the connection between the increase in temperature and dengue incidence^{226,236,237}. These studies also arrived concluded that relative humidity is also an important factor in dengue's dynamics as well^{226,238}.

4. THE IMMUNE RESPONSE AGAINST DENGUE VIRUS

During their life span, animals encounter a wide variety of pathogenic viruses and microorganisms. The immune system constitutes the defence line that protects these hosts from otherwise lethal or compromising infections.

In the case of viruses, prevention of infection or viral clearance depends on both innate and adaptive immune response mechanisms, where the humoral immune responses (mainly antibodies) are able to prevent the infection of cells at the earliest stage, while the cellular component of immunity is more involved in the elimination of the viral infection once it has been established²³⁹.

Dengue virus is a particular case among human pathogens in that the immune response of the host may direct the clinical outcome of the infection in two completely opposite directions: it can either increase the severity of the disease or it can efficiently neutralise the pathogen¹. This appears to be the result of a combination of unique viral characteristics, more importantly, the existence of four serotypes that share many common antigens but are different enough to avoid cross-neutralisation, and their tropism toward the mononuclear phagocytic cell line which is one the main components of the immune system²²⁰.

4.1. Innate Immunity

The innate immune system is the first line of defence against viral infections and involves the recognition of pathogen-associated molecular patterns (PAMPs) within infected cells that trigger a variety of immediate defensive measures²²⁰. The main characteristics of innate responses are that they do not require previous exposure to the pathogen for activation and don't show enhancement upon subsequent responses. Some examples of innate immune mechanisms are the Toll-like receptors which can signal cells to produce different cytokines, the complement system and natural-killer cells²³⁹.

4.1.1. Toll-like receptors (TLRs) and other intracellular sensors

TLRs are important pattern recognition receptors that, together with other intracellular proteins, like the helicases melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene 1 (RIG-I), act as sensors to mediate some of the first innate responses against viral RNA^{240,241}. TLR3, RIG-I and MDA5 have been shown to recognize the viral genome during the entry process and modulate viral replication by inducing a strong Interferon- β and IL-8 response^{240,242}. Other TLRs involved in a similar way are TLR7 and TLR8 which are also able to recognize viral RNA molecules and induce a similar cytokine response to prevent viral amplification^{243,244}. Upon stimulation of these receptors, the activation of an intracellular signalling pathway leads to an increase production of proinflammatory cytokines that compromise viral replication within the cell²⁴⁵.

4.1.2. Interferons (IFNs) and cytokine response

IFNs can be divided in three different types on the basis of their biochemical characteristics, pattern of expression and mechanism of action, in the case of DENV type

I and type II IFNs are particularly important²³⁹. Type I IFNs like IFN α and IFN β are produced by a wide range of cell types and have antiviral activity against DNA and RNA viruses²²⁰.

After dengue virus infection, IFNs are rapidly induced and promote a general antiviral state within the infected cell by activation of RNA nucleases, stimulation of lymphocytes and activation of other effector cells²²⁰. Several studies have shown that production of IFN α and IFN β have an inhibitory effect on dengue virus infection, especially during the initial stages of the RNA replication, and that these types of innate responses are important to control the infection *in vivo*^{246,247}.

IFN γ is a type II IFN and is produced mainly by T lymphocytes and natural-killer cells in response to the interaction with proteins derived from dengue virus (not as a result of viral infection)²³⁹. The function of this cytokine can be either beneficial or detrimental for the host depending on the scenario: there are studies indicating that IFN γ can prevent the infection and spread of dengue virus^{246,248,249}, while others state that it can actually promote infection by increasing the expression of the Fc γ receptor in monocytes through an antibody-dependent mechanism²⁵⁰.

In response to these antiviral effects, dengue viruses have developed mechanisms to block IFN's antiviral activities with NS2A, NS4A, NS4B and NS5 showing the ability to inhibit the IFN-induced gene expression^{133,173}.

4.1.3. The complement system (C')

The C' is considered one of the main effectors of the innate immune systems although it also has the ability to interact with the adaptive branch of immunity²⁵¹. This system consists of a network of proteins able to recognize target proteins and initiate an amplifying chain-reaction type activation that leads to the lysis of infected cells or even enveloped viruses like dengue²⁵². In addition, C' can also mediate the neutralization of viral particles reducing the levels of infection^{251,253}.

However, dengue virus interactions with the proteins of C' are thought to play an important role in viral pathogenesis, particularly in the process of vascular permeability through the viral protein NS1 that has been shown to regulate C' function by degrading some of its components^{110,117}.

4.1.4. Natural killer (NK) cells

NK cells are a specialised type of cytotoxic lymphocytes that have an analogous function in the innate immune system to that of the CD8+ T lymphocytes in the adaptive immunity. These cells represent between 5-15% of circulating lymphocytes and are able to rapidly kill cancer-prone or virus-infected cells²²⁰.

The cytotoxic activity of NK cells does not depend on the specific recognition of viral peptides, but rather on the detection of an “altered cell” phenotype through the balanced response involving a variety of stimulatory and inhibitory receptors on the surface of the cells. NK cells are particularly sensitive to a reduced expression of major histocompatibility complex (MHC) protein and their lytic activity is greatly enhanced by the presence of IFNs in the cellular milieu²⁵⁴. Moreover, antibodies bound to cell surface proteins mediate antibody-dependent cellular cytotoxicity (ADCC) through the Fcγ receptors on the NK cell²²⁰.

In the case of dengue infection, cytotoxicity of infected cells by NK cells in an ADCC manner has been demonstrated²⁵⁵. Although ADCC seems to be the main lytic mechanism involved in clearance of infected cells, NK cells are also able to perform this function in the absence of antibodies which implies the existence of a more direct way of activation; in fact, NK cell surface protein NKp44 has been implicated in the direct recognition of dengue E-derived peptides, although its actual role in NK cell function during dengue virus infection needs further confirmation^{256,257}.

In an attempt to counteract NK cell function, dengue viruses can cause an increased expression of MCH-I antigens on the surface of infected cells making them less susceptible to NK cell mediated lysis²⁵⁸. In spite of this, the role of NK cells on the defence against dengue virus infection *in vivo* is still highly unknown.

4.2. Adaptive Immunity: Lymphocytes

When the pathogen is able to surpass the defence established by innate immunity, it generates a threshold level of antigens and stimulatory signals that triggers the activation of the adaptive immunity²⁵². Opposite to the innate mechanisms, adaptive immunity refers to responses that develop after a first exposure to the pathogen and act in an antigen-specific way, showing enhancement of the response in terms of time, affinity and intensity after subsequent encounters²³⁹. The adaptive immune response is divided into two major components: the humoral immune response involves the activities of B lymphocytes and antibodies, while the cellular responses cover the functions of the CD4+ and CD8+ T lymphocytes.

4.2.1. Targets of the T cell response against dengue virus

Primary infection with dengue virus induces the response of very diverse virus-specific T cells in which some are specific against the infecting serotype while others are heterologous and show cross-reactivity against other serotypes and related flaviviruses²³⁹.

As shown in Fig. 19, although the viral epitopes responsible for inducing CD4+ and CD8+ T cell-mediated immunity are found throughout the viral polyprotein, most of them

are concentrated on the non-structural proteins, especially on NS3 and NS5, which represent the main target of the adaptive cellular response^{259,260}.

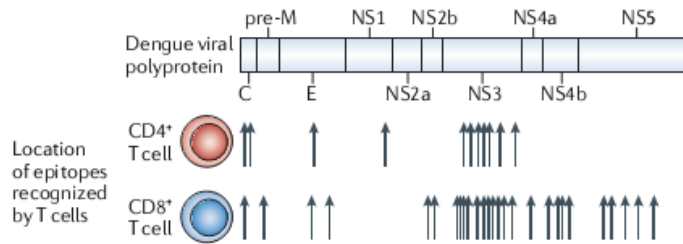


Figure 19. Dengue virus protein targets to T cell response. Scheme showing the general structure of the dengue virus polyprotein indicating (with arrows) the position of epitopes recognize by CD4+ and CD8+ human T cells (Reprint from reference 260).

Given that NS3 protein represents a small fraction of the viral genome and that there's a $\approx 70\%$ sequence identity between the four dengue serotypes, T cell epitopes are highly conserved and generally induce cross-reactive immune responses²⁶⁰. Extending this idea to the whole viral genome we can conclude that, in general, an adaptive immune response directed against a non-structural protein will show a great degree of cross-reactivity while responses against structural proteins, which show a higher variation among DENV serotypes, tends to be more type-specific²³⁹.

4.2.2. CD4+ T lymphocytes

After recognising virus infected cells, dengue virus-specific CD4+ T cells are activated and respond with a diverse range of functions that include: proliferation, cytokines (CKs) production and target cell lysis⁵³.

Although these cells have demonstrated the capacity to lyse infected cells *in vitro*, CD4+ T cells principal response during a dengue infection involves the production of a wide range of CKs. Most studies agree that the pattern of CKs produced in these contexts follows a TH1- or TH0-like pro-inflammatory type I CKs profile, like tumour necrosis factor α (TNF α), IFN γ and interleukin 2 (IL2)²⁶¹. In addition, CD4+ T cells activated against dengue virus have been shown to express Fas-ligand and perforin which might be involved not only in direct killing of infected cells but also on tissue damage, thus contributing to the pathogenesis of the infection²⁶².

It's important to mention that the aminoacid differences between dengue viruses can affect the interaction between the viral-derived peptide-MHC complexes on the infected cells and the T cell receptor (TCR) of the lymphocyte, which could have significant functional consequences, since the type of response elicited by T cells is highly dependent on the strength of the signal sensed through the TCR²⁶⁰. Especially during a secondary heterotypic dengue infection, memory T cells created during the first infection

may not interact properly with the peptides of a different serotype, inducing an abnormal response that could influence the clinical outcome of the infection⁵³.

4.2.3. CD8+ T lymphocytes

Like CD4+ T lymphocytes, upon primary infection with dengue virus, specific CD8+ (cytotoxic) T lymphocytes (CTLs) generate responses specific to the infective serotype and others that cross-react with heterologous serotypes^{239,263}.

Although their role during dengue infection is not very clear, some studies have shown evidence supporting the idea that activated CTLs have an important role in controlling the infection²⁶⁴. Activation of CTLs happens after recognition of the specific epitope in the context of MHC-I molecules which induce IFN γ production, clonal proliferation and cytolytic activity²⁶⁵. Interestingly, some epitope variants derived from heterologous viral serotypes have been shown to alter the proportional response of CTL by acting as superagonists causing an effect that may contribute to the pathologic manifestations of the infection; this is more relevant given the fact that CTLs responses against dengue virus are frequently cross-reactive^{239,266}.

In summary, since T lymphocytes recognise virus-derived peptides in the context of MHC molecules and are not able to recognize complete viral particles, their main role is not in preventing viral infection. Conversely, through their diverse responses after activation, specific T cells could restrict viral replication and the development of the disease by eliminating infected cells and producing proinflammatory CKs²⁶⁰.

T cells have also been involved in the pathogenesis of the severe manifestations seen during dengue virus infection, mainly as a result of the proinflammatory effects of the T-cell derived CKs. However, it's still unclear to what extent is T cell activation involved in either protective immunity or pathogenic processes²⁶⁷.

4.3. Adaptive Immunity: Antibody response

Neutralising antibodies against viruses are widely considered to be vital for controlling infection and dissemination of the pathogen; moreover, they are the most common element used to indicate protection against most viral diseases^{53,220}. These statements apply also to the infection with dengue, although in this case the correlation is not always clear²²⁰.

4.3.1. Antigenic structure of dengue virus and antibody specificity

After an infection with dengue virus, both human patients and murine models have been shown to developed antibodies against structural and non-structural viral proteins. Proteins E, PrM and NS1 are the main targets of the antibody response while antibodies

against C, NS3, NS4b and NS5 have also been described although they are not as common or abundant²⁶⁸⁻²⁷⁰.

4.3.1.1. The envelope protein

E protein is the most important target of the antibody response during infection. Neutralising epitopes have been described on all three domains displaying varying degrees of cross-reactivity not only between dengue serotypes, but also among other flaviviruses^{64,260}. Several reports indicate that antibodies against DIII, particularly those that recognise the upper lateral surface of the domain, show the highest neutralizing capacity; coincidentally, this is also the most variable domain between serotypes, which means that these antibodies are usually highly specific and explain why DIII has been considered for vaccine development^{92,271,272}.

However, studies have shown that after natural dengue infection, the immune response is dominated by highly cross-reactive, weakly neutralising antibodies, directed mostly against epitopes on DI/DII^{273,274}. While the FL appears to be a major target of the antibody response²⁷⁵, there are reasons to think that the contribution of DIII is very limited and does not account for the strong type-specific neutralising response seen in patients: levels of anti-DIII antibodies in human sera are very low²⁷⁶ and depletion of DIII-specific antibodies has little effect on the viral neutralizing capacity of human sera²⁷¹. Moreover, viruses carrying mutations in DIII epitopes recognised by neutralising antibodies were still efficiently neutralised by human immune sera²⁷⁷.

While enhancing cross-reactive epitopes seem to be simpler in structure, recent data indicates that the majority of neutralising antibodies present in sera from infected patients recognise complex quaternary epitopes that are only present on the surface of the intact virion^{278,279}. In fact, the symmetric organisation of E proteins on the viral surface, together with the curvature imposed on E by restriction of the membrane attachment, creates unique quaternary and conformational epitopes involving two or more E molecules that are targeted by strong neutralising antibodies²⁷⁵. Of note, some of these antibodies bind quaternary epitopes that are formed on the herring-bone configuration of E and are thus restricted to the viral surface: monoclonal antibody (mAb) HM14c10 binds an epitope present between two adjacent E dimers of DENV1²⁸⁰, while mAb 5J7 footprint involves critical interaction with 3 adjacent E monomers²⁸¹. On the other hand, mAb 1F4 epitope is restricted to a single E monomer of DENV1 but on a particular conformation that exist only within the context of the viral particle and not on the recombinant protein²⁸². Binding of epitopes located at the DI/DII hinge region is a common feature of these mAbs, indicating that interference with this region has highly neutralising potential²⁸³.

Recently, a new group of strongly neutralising antibodies that target complex quaternary epitopes was described. These mAbs target epitopes restricted to the interface

between two E proteins in a head-to-tail dimer conformation and therefore do not require higher-order arrangements of E like mAbs HM14c10 and 5J7²⁸⁴. MAb 2D22 for example, binds to the dimer interface but its epitope involves significant interactions with residues on DIII, which is why it only recognises DENV2²⁸⁵. In contrast, a recent group of mAbs isolated from previously infected patients target the valley formed between the E monomers; specifically, these antibodies target conserved residues on the DI/DIII-DII dimer interface^{284,286}. This epitope, named EDE (E-dimer epitope), overlaps the highly conserved region of interaction with PrM which explains why these antibodies are able to induce strong neutralisation of all 4 serotypes²⁸⁶. The binding determinants of these EDE mAbs involve the FL and the loops in its close vicinity within the context of the E dimer and were further classified in two groups: EDE1 mAbs were shown to improve binding in absence of the Asn-153 glycosylation, while EDE2 mAbs showed better binding in presence of the glycan²⁸⁴. Moreover, a recent study demonstrated that EDE1 antibodies are also able to neutralise ZIKV, EDE2 antibodies showed less affinity towards ZIKV²⁸⁷⁻²⁸⁹.

4.3.1.2. PrM protein

Antibodies against this protein seem to be stimulated by the Pr peptide released from the viral surface once the maturation process is complete. In addition, the presence of immature viral particles as a result of incomplete cleavage of the Pr peptide can also stimulate the response against this protein⁷⁸.

PrM antibodies are highly cross-reactive among the different serotypes and show very little neutralizing capacity^{78,260,290}. Instead, increasing amounts of evidence supports the notion that anti-Pr antibodies can actually favour the infection process by promoting the internalization and infection of immature viruses^{102,291,292}.

4.3.1.3. NS1 protein

As mentioned before the NS1 glycoprotein can be found attached to the surface of infected cells and is also secreted from them as a hexamer. This explains why, in spite of being a non-structural protein, it's among the significant targets of the antibody response¹¹⁰. When compared with antibodies induced against the E protein or PrM, antibodies against NS1 show restricted cross-reactivity between the four serotypes²⁹¹.

Beside antibodies that cross-react with other dengue serotypes, the antibodies produced during viral infection have the capacity to recognise host proteins. Antibodies against E protein obtained from infected patients were shown to recognise human plasminogen and fibrinogen, while there is evidence suggesting the existence of molecular mimicry between NS1 and several host protein in a way that anti-NS1

antibodies are able to cross recognize human plasminogen, coagulation factors, platelets and endothelial cells and elicit responses that might be implicated in the pathogenesis of infection^{122,293,294}.

4.3.2. The antibody response upon Dengue Virus infection

Since earlier studies on the course of infection and immunity of dengue, it was demonstrated that after the first infection with any of the four serotypes, patients showed long-lasting protection against reinfection with the same serotype, but only short term protection when challenged with heterologous serotypes²⁹⁵. Moreover, after this period of time, patients suffering subsequent heterotypic DENV infection showed higher incidence of dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) compared to the cases present during primary infection²⁹⁵. An antibody-dependent enhancement of infection (ADE) was initially proposed to explain this phenomenon, which is explained in detail below^{53,270}.

Chang *et al* gave compelling evidence to explain the short-term broad neutralising phase of the immune response against dengue²⁹⁶ showing that soon after the infection with a particular serotype, patients sera contained high concentrations of weakly neutralising antibodies that were able to form large aggregates when facing a different viral serotype, thus preventing viral infection. This type of protection is temporal because it depends on a high threshold level of cross-reactive antibodies that diminishes in time.

The differences seen on the immune response against a different dengue serotype during a secondary infection are the result of the pre-existing heterotypic immunity in which the second response occurs: when facing the new serotype, cross-reactive B and T lymphocytes are able to respond faster than naïve immune cells producing an anamnestic response to the antigens that are shared between the previous and the infecting serotype²²⁰. Since this early response to the secondary is based on cross-reactive antigens, it shows lower affinity and appears to be less efficient in neutralising the virus responsible for the initial infection, a feature known as “original antigenic sin”²⁶⁰. In time, the neutralising response against the second serotype increases leading to a protective long-lasting response that includes serotypes that have not been previously encountered by the individual which explains why tertiary infection with dengue is rare²⁷⁰. Indeed, T cell response has been shown to favour the selection of cells that recognise cross-reactive epitopes²⁹⁷, while in the case of B cells, type-specific cells show a steady reduction from the time of infection in such a way that, by the time of the secondary infection, mainly cross-reactive cells are present²⁹⁸⁻³⁰⁰.

The kinetics of the antibody response has also been studied. During the primary infection anti-dengue IgM antibodies (predominately directed against the E protein) appear 4-5 days after the onset of fever and remain for up to three months following the

infection. IgG antibodies are detectable a couple of days after the IgM, their titre peaks several weeks after the infection process has ended and then declines to levels that remain stable for decades²²⁰. During the first infection, the IgG antibodies produced show specificity against PrM and E and belong mainly to the IgG1 and IgG3 subclasses, which indicates a bias towards a proinflammatory response^{270,301,302}. During a secondary infection, the memory B-cells from the primary infection respond with an acute rise in IgG titres that are detectable on the first day of fever, while IgM response varies and in some cases is undetectable; additionally, antibodies against the non-structural proteins of the virus are more likely detected during secondary infections²⁷⁰.

Regarding specificity, several studies have shown that antibodies elicited after infection are directed mainly against the E protein and to lesser degree against PrM and NS1. However, even though the results across studies are highly variable, contrary to the response during primary infections which is strongly dominated by E, antibodies induced in a second infection show a broader range of recognition with an increasing percentage of antibodies directed towards PrM and NS1, which points to a fundamental difference between primary and secondary dengue infections^{273,303}.

4.3.3. Mechanisms of protection

There are several mechanisms through which antibodies can elicit their protective function against viral infection: opsonisation, targeting infected cells for elimination and neutralisation.

4.3.3.1. Opsonisation of pathogens

In this mechanism, antibody molecules bind to the surface of the viral particles and targets them for elimination by lysis through C', or by phagocytosis in Fc γ receptor-bearing phagocytic cells. This is a very efficient method to eliminate pathogens that are not able to replicate in phagocytic cells; however, in the case of dengue, the small size of the virions limits the lytic function of the complement system and the opsonisation (that is, antibody binding below the threshold required for neutralisation) would actually favour the infection process since it facilitates the uptake of the virus by its target cell via ADE as mentioned previously²²⁰.

4.3.3.2. Targeting of infected cells for elimination

As opposed to what happens during opsonisation, in this mechanism antibodies bind to infected cells that express viral antigens on their surface. Like in opsonisation, the binding of antibodies recruits lytic agents like C' or NK cells, CTLs and macrophages that could kill the infected cell through ADCC²²⁰.

Several studies indicate that this mechanism can provide partial protection against the infection and is mediated by antibodies directed against non-structural viral proteins, mainly NS1 and NS3^{304,305}

4.3.3.3. Neutralisation

This last mechanism is also considered the most important in determining protection against most viruses. In the case of dengue, the E protein is the main antigen involved in the generation of protective antibodies²²⁰.

For flaviviruses in general, neutralisation of virions follows the “multi-hit” requirement model in which neutralisation happens when an individual viral particle is bound by a critical number of antibody molecules that surpass a determined threshold^{306,307}. This threshold is different for each epitope and depends mainly on a combination of two biochemical factors: antibody affinity and the accessibility of the epitope on the virus²⁷².

Antibody affinity refers to the strength of binding between an antibody molecule and its antigen, and determines the fraction of the available epitopes on the viral surface that are occupied by the antibody at any given concentration of the latter³⁰⁸. Affinity usually correlates with neutralising activity since antibodies with higher affinity for their epitope are able to reach the neutralization threshold even at lower concentrations³⁰⁹. However this is not always the case, as the number of available epitopes required for neutralisation might be very high or even impossible to reach regardless of the antibody binding affinity.

While affinity determines the relative occupancy of epitope by an antibody, antigen accessibility determines the total number of epitopes that are available for binding on the viral surface under any particular condition³⁰⁷. Thus, epitopes that are highly exposed may exceed the neutralization threshold with a low affinity antibody, while poorly expressed sites could need highly or complete occupancy to achieve neutralization²⁷². In the case of dengue, several factors regulate epitope accessibility on the viral surface:

1. Steric constraints: As a consequence of the dimeric conformation of the E protein and its dense arrangement on the viral surface, not all of the antibody epitopes are equally accessible for binding and antibody recognition is also limited, which means that, for some epitopes, it is not possible to exceed the neutralization threshold at any antibody concentration^{260,307}.
2. Structural dynamics of E: As explained in previous sections, the E protein is a highly dynamic structure that is not only in constant movement due to the slight conformational changes involved in viral “breathing”⁴⁸, but also suffers changes in its oligomeric state during the viral life cycle and in response to temperature and pH²⁶⁰. This directly influences the neutralization capacity of antibodies as the epitopes on the viral surface are highly dependent on these factors³⁰⁷.

3. Steric interference of the antibody molecule: The space occupied by an antibody molecule is considerably larger than the surface of the paratope it recognises, which limits the amount of molecules that are able to bind to the viral surface and may also interfere with the accessibility to other closely located epitopes³⁰⁸.

Regarding their function, there are several mechanisms by which antibodies can neutralise flavivirus virions, all of them involving the steps in the entry process to the host cell. As depicted in Fig. 20, some antibodies, prevent infection by actively blocking attachment of the virus to the host cell, or promoting viral detachment from cells. A different type of neutralising antibodies act downstream of the binding step by interfering with the conformational changes of the virus surface required for membrane fusion³¹⁰. In fact, structural analyses indicate that the strong neutralising capacity of antibodies against quaternary epitopes derives from their ability to interfere with viral surface dynamics: As mentioned before, DENV fusion with the endosome requires a reorganisation of the viral surface in which the E dimers disassociate and form trimeric structures; binding of antibodies to complex quaternary epitopes that span between 2 or more E monomers, may inhibits trimerisation by locking the E monomers into a fixed position, thus preventing viral escape from the endosome^{281,284,285}. This mechanism of action has also been described for antibodies directed against the DI/DII hinge region^{272,282}.

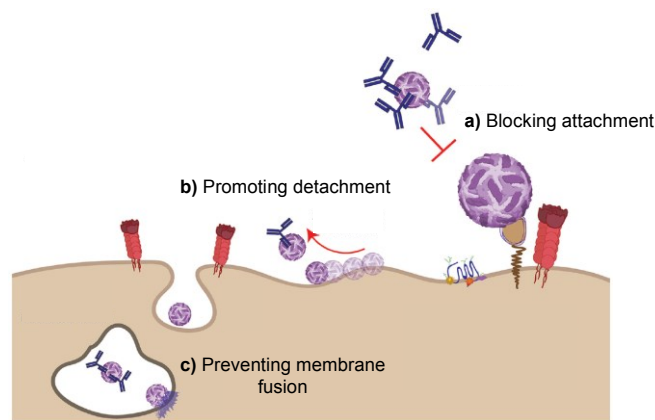


Figure 20. Mechanisms of antibody-mediated neutralisation. Antibody-mediated neutralisation of flaviviruses may be achieved by interfering with critical steps of the viral cycle preceding the release of the viral genome in the host cells, such as **a)** blocking viral attachment to the cell membrane by preventing interaction with cell receptors, **b)** promoting detachment of the viral particle before the clathrin-mediated endocytosis and **c)** by inhibiting the process on viral fusion with the endosome membrane. (Adapted from reference 310).

4.3.4. Antibody-dependent enhancement of infection (ADE)

As mentioned before, ADE describes a situation in which there is a significant increase in the efficiency of viral infection in presence of non-neutralising concentrations of antibodies (mainly cross-reactive antibodies) against the virus³⁰⁷. This event is the

leading theory to explain the data showing that, after a period of broad cross-protection following a primary infection with dengue, a secondary infection with a different serotype carries higher risk of developing severe haemorrhagic manifestations of the disease²⁷⁰. Moreover, this risk has been shown to increase as the time between the primary and secondary infection increases^{27,311}.

Although it has been hypothesised for many years³¹², early studies in Thailand pointed out that the DHF/DSS cases were found mainly in two groups: first-time infected babies born to immune mothers, and children with a secondary infection³¹². Observations made from a series of studies from Cuba provided new data to further support the effect of this phenomenon in dengue pathogenesis: before 1990, the Cuban population had only experienced 2 outbreaks of dengue following the introduction of serotype 1 in 1971 and serotype 2 in 1981. After that, the strategies to control the vector prevented the transmission of the virus until 1997 when the serotype 2 was reintroduced in the capital; in this outbreak, the cases of severe dengue were restricted to patients old enough to live through the serotype 1 outbreak in the 70's, a situation that was confirmed for all cases after serologic analyses³¹³. The ADE concept was born to explain the association between secondary infection and severe disease, and has been described for several viruses apart from dengue³¹⁴⁻³¹⁶.

In vitro, all the antibodies against the dengue virion have the capacity of eliciting ADE when used at a concentration below the threshold for neutralisation^{270,309}. In these conditions, antibodies bind to the viral surface in a weak opsonic-like manner that allows the interaction of the virus with Fc γ receptor-bearing myeloid cells like monocytes and macrophages, where the infection occurs after the endocytosis of the complex³¹⁷. Contrary to a regular infection, in ADE there is significant increase in viral production which results in a higher viremia and more severe symptoms³¹⁸. This could be the result of an increased number of infected cells due to the alternative route of infection²⁹⁵. In addition, it has been demonstrated that infection through the Fc γ -receptor down regulates the antiviral response of phagocytic cell by enhancing IL-10 production and other anti-inflammatory CKs^{319,320}, and inhibiting the production of IFN γ and TNF α ³¹⁹. In contrast, a recent study in macrophages failed to find a diminished intracellular antiviral response, but rather observed increased membrane fusion activity within infected cells, a feature associated with increased viral production³²¹.

Besides the cellular-related mechanisms for enhancing infection, cross-reactive antibodies have been shown to allow the infection of immature viral particles. Available data indicates that immature viruses are not able to interact properly with the host-cell receptors, but are able to bypass this requirement through the interaction with antibodies and Fc γ -receptors; once inside the cell, uncleaved PrM is efficiently processed allowing viral infection²⁹².

In fact, accumulated evidence indicates that the cross-reactive antibodies targeting structural proteins on the viral surface, especially those against PrM and FL, are the main serum component responsible for ADE *in vivo* and *in vitro*²⁷⁹. Previous studies have confirmed the possibility of ADE, not only among DENV serotypes, but also among heterologous flaviviruses; which is expected given that the epitopes recognised by highly cross-reactive antibodies are also conserved in other viruses³²²⁻³²⁴. Models done considering the effect of ADE on viral dynamics suggest that this form of enhancement provides a fitness advantage that naturally selects strains that undergo enhancement over those that do not³²⁵.

Although there is a significant amount of evidence supporting the ADE theory in humans and in animal models²⁹⁵, its true impact in dengue infection has not been fully elucidated and its role in severe haemorrhagic disease remains circumstantial²⁹⁵. Instead, several observations indicate that there are other elements involved in the severe pathogenesis of dengue²⁶⁰:

1. DHF and DSS can occur during the first infection with dengue, in absence of enhancing antibodies.
2. Even though high viremia and plasma leakage are usually correlated, there is no causality link between increased viremia and the occurrence of haemorrhagic manifestations of the infection.
3. Some studies show that there is no difference in the ADE activity of pre-infection sera from patients who did and did not develop severe dengue disease after secondary infection.
4. As mentioned before, recently a group of laboratories was able to demonstrate the role of NS1 in vascular permeability¹²⁵, an antibody-independent mechanism that might be important to initiate the haemorrhagic manifestations of the disease.

Among the mechanisms that could contribute to the development of DHF/DSS are: C' activation by immune complexes, induction of autoimmunity by molecular mimicry of the virus, existence of highly pathogenic viral strains and overstimulation of T lymphocytes leading to a pathogenic cytokine storm²⁶⁰.

4.3.4.1. Fcγ Receptors (FcγRs)

FcγRs are a family of glycoproteins found on the surface of haematopoietic cells and serve important functions during maturation, cell activation and phagocytosis¹⁹⁴. Humans express three types of FcγRs: FcγRI (CD64), FcγRII (CD32, subdivided in FcγRIIa and FcγRIIb) and FcγRIII (CD16, also divided in FcγRIIIa and FcγRIIIb) that vary in terms of binding affinities to IgG and type of signal transmitted upon stimulation¹⁹⁴. For example,

Fc γ RI is the only one capable of binding monomeric IgGs, while Fc γ RIIb is the only one that induce an inhibitory signal³²⁶.

As previously mentioned, dengue virus primarily targets cells from the macrophage-monocyte lineage that express Fc γ Rs on their surface. Although both Fc γ RI and Fc γ RIIa are able to mediate the internalization of viral particles bound to IgG, Fc γ RIIa has proven to be more relevant since it's more widely expressed, binds all IgG subclasses and is more efficient in mediating the antibody-mediated uptake^{327,328}. In contrast, Fc γ RIIb stimulation inhibits Fc γ R-mediated phagocytosis thus preventing ADE³²⁹.

Integrating new data regarding the neutralization and enhancing capacity of antibodies against different viral proteins into the design of immunogens, could benefit greatly the development of vaccines and therapeutics against dengue. In the case of vaccines, a major goal is to engineer antigens with the capacity of steering the humoral immune response away from poorly neutralizing, cross-reactive and enhancing epitopes in order to target the ones able to elicit highly neutralizing antibodies against all four serotypes²⁷².

5. VACCINES AGAINST DENGUE VIRUS

The efforts to develop vaccines against other members of the Flaviviridae family have rendered efficient compounds for YFV, JEV, TBEV and others³³⁰⁻³³³. However, despite the considerable amount of work made over the years²²⁰, the development of an efficient vaccine against dengue virus is still unresolved and faces many challenges that severely compromise any progress that has been made in the field:

1. The existence of four different serotypes of dengue virus, together with the potential detrimental effect of the immune response via ADE, impose the requirement for any viable vaccine candidate to generate a balanced, long-lasting neutralizing response against all four serotypes at the same time³³⁴⁻³³⁶.
2. The lack of an adequate animal model limits the strength of the data obtained for each vaccine candidate during preliminary testing, since there's poor correlation between the course and the immunity of the infection when compared to humans. Initially, animal models for dengue infection were limited to the demonstration of immunogenicity in mice and non-human primates (NHP)^{337,338}. However, the AG129 mouse model (IFN- α/β and γ receptor deficient) has been shown to sustain DENV2 replication and suffers from vascular features similar to those present in humans with haemorrhagic manifestations of the infection, thus allowing further data to be derived from *in vivo* experiments^{339,340}.
3. The fact that the pathogenesis of the disease is still not fully understood limits the process of testing the vaccine candidates to assess their immunogenicity and

possible side effects, thus increasing the risk of eventual clinical trials²²⁰. Moreover, although it is well known that antibodies play a major role in protection against the infection³⁴¹, there're still many unanswered questions regarding the quantity or quality of antibodies needed to achieve protection³⁴².

4. The epidemiology of the disease directly affects the formulation requirements of the vaccine since it should take into account the target population. In endemic areas, there is an urgent need for routine vaccination of children, so the vaccine should be safe and compatible with other childhood vaccines. In addition, due to the fact that nearly all endemic countries are underdeveloped, the vaccine should also be inexpensive and stable. In contrast, vaccines aimed for international travellers could cost more but require a short vaccination protocol⁵³.

The first efforts to develop a vaccine against dengue started 50 years ago with significant advances occurring in the last 10 years. Several vaccine candidates are currently at advanced stages of development and show promise in clinical studies; on top of that, the understanding of the human immune response against dengue virus and, more importantly, the notion of what constitutes a protective response against this pathogen is increasing^{334,336}. Presently, the vaccine candidates under development cover a wide range of approaches including: live attenuated virus vaccines, molecularly attenuated live virus vaccines, live chimeric virus vaccines, inactivated virus vaccines, recombinant subunit vaccines and genetic (DNA/RNA) vaccines.

5.1. Live attenuated virus vaccines

Live attenuated virus (LAV) vaccines represent the most common type of commercially available viral vaccines (including yellow fever, measles, mumps, influenza, varicella and polio) and are composed of weakened viruses that are still able to induce a long-lasting adaptive immune response (both cellular and humoral) by mimicking the natural infection and exposing the same antigens that a wild type virus do during a normal infection, but with a diminished replication and infective potential in order to avoid pathological effects³⁴³.

In the case of dengue, the first attempts to develop a LAV vaccine were in 1929 with major advances happening after the second world war when Sabin and Schlesinger were able to obtain the first attenuated strain of dengue virus (Dengue virus 1 and Dengue virus 2, respectively) through serial passages in mouse brain²²⁰. After figuring out the process to achieve attenuation of the viral strains, a number of groups produced and tested different attenuated strains, however they all failed in proving seroconversion against all four serotypes and adequate safety, with reports of unacceptable reactogenicity related to some of them³⁴⁴⁻³⁴⁷. Moreover, achieving a balanced response against all serotypes has

proven to be complex obstacle for LAV vaccines, as immune interference between the different viruses of the formulation is common³⁴⁸.

Further safety issues inherent to LAV vaccines are: the risk of reversion from the attenuated phenotype back to its original virulence, uncontrolled variation in the attenuated strain during culture passages, the risk of developing ADE due to weak or unbalanced immune responses, the risk of severe side effects in immunocompromised patients as in those with pre-existing immunity, and the risk of spreading the vaccination strains²²⁰.

5.2. Molecularly attenuated live virus (MALV) vaccines

Contrary to classic attenuation methods, MALV vaccines are based on obtaining attenuated variants of wild-type strains by means of conscious genetic alteration, which allows for the generation of vaccine candidates in a direct and controlled manner, circumventing many of the safety issues related to LAV vaccines, particularly those involving the risk of reversion to wild type virulence²²⁰.

The work of C.J. Lai and colleagues to develop an attenuated variant of Dengue virus 4 represents one of the efforts of vaccine development using MALV technology³⁴⁹. In this particular case, the attenuation consisted in a large 30nt deletion in the 3' UTR (named DENV4-Δ30), and initial results of Phase 1 trials revealed that the virus was not only immunogenic but also stable and well tolerated by the subjects although it only involved one serotype³⁵⁰.

In collaboration with the Novartis Institute for Tropical Research, The Agency for Science, Technology and Research in Singapore developed a DENV2 MALV vaccine candidate that lacks NS5's MTase activity, which eliminates the ability of the virus to block the IFN response within infected cells and is highly attenuated in animal models³⁵¹. Initial results showed that a single dose of the vaccine was able induce a 100% of seroconversion and protect NHPs from viremia. In addition, the virus is not able to replicate in mosquitos, which eliminates the risk of spreading the attenuated strain³⁵¹.

Using another approach, researchers from Arbovax developed attenuated strains for all 4 DENV serotypes by altering the transmembrane domain of E and selecting for mutants that replicate well in insect cells but not in mammalian cells. The tetravalent vaccine obtained using these host range mutations (HR-tet), was able to induce a balanced response with 100% seroconversion in African green monkeys after a single dose³⁵².

5.3. Live chimeric virus (LCV) vaccines

As an extension from the MALV vaccines, LCV vaccines also involve genetic manipulation of the viral genome but in this case, the vaccine is composed of live (generally

attenuated) viruses that are commonly used as a backbone to express antigens of another virus³⁵³. One of the first efforts in this field came from the same group of C.J. Lai by using the DENV4-Δ30 strain as a background for generating new chimeric viruses through the substitution of the genes coding for all (C-PrM-E) or some (PrM-E) of the structural proteins with the those of the other dengue serotypes, thus generating DENV-1/DENV-4 and DENV-2/DENV-4 chimeras with the DENV-1 or DENV-2 antigenicity but the attenuated properties of the DENV4-Δ30 strain³⁵⁴. This exemplifies the advantage of the LCV vaccines: retaining the desired markers or properties of a particular strain while changing the immunogenic properties of the viral particle²²⁰.

Currently the US National Institutes of Health are developing two tetravalent LCV vaccines based on the DENV4-Δ30 attenuated strain called TV003 and TV005³⁵⁵. These formulations were made by incorporating the 3'UTR Δ30 attenuations on wild-type strains using a combination of MALV and chimeric approaches to achieve an efficient type-specific strains to include in the tetravalent formulation³⁵⁶. So far, the vaccine has demonstrated to be safe in humans³⁵⁷ and elicits a robust and balanced tetravalent response after a single dose with over 90% tetravalent seroconversion in naïve-recipients³⁵⁸. The vaccine has been licensed to several manufacturers and several Phase 2 and Phase 3 studies are currently on-going³⁵⁶.

Another case of LCV is TDV (formerly known as DENVax), a tetravalent vaccine based on the LAV DENV2 candidate attenuated by serial passage in primary dog kidney cells (DEN-2 PDK-53)³⁵⁹. In this case, the tetravalent formulation was achieved by replacing the *PrM-E* genes of the DEN-2 PDK-53 strain, with those of DENV1, DENV3 and DENV4³⁶⁰. After confirming safety, immunogenicity and efficacy on preclinical studies³⁶⁰, several Phase 1 and Phase 2 studies have evaluated the efficiency of the vaccine in a variety of setting using different tetravalent formulations and routes of administration. The data demonstrated that the vaccine is able to induce adequate seroconversion in naïve individuals and Phase 3 trials are expected to begin in the near future³⁶¹⁻³⁶³.

Sanofi-Pasteur's Dengvaxia® (CYD-TDV), is the most advanced formulation for vaccination against dengue in production, and is now available for licensing in several countries around the world³⁴². CYD-TDV is a LCV vaccine that uses the 17D attenuated strain of Yellow Fever virus as a backbone to express the *prM* and *E* genes of each Dengue serotype³⁶⁴. Although proving to be stable and highly immunogenic in preclinical phases, the safety of tetravalent formulation was confirmed in several Phase II studies, however an unbalanced and low neutralizing response (when compared to animal models) was noticed once the data was analysed³⁶⁵⁻³⁶⁷. The vaccine was then tested in two Phase III trials (Asia and Latin-America), although showing adequate seroconversion rates in vaccinated patients, the results indicated that the vaccine induced an unbalanced

response against the different serotypes with DENV4 and DENV2 showing the highest and lowest efficacy (76.9% and 43%, respectively)^{368,369}. Moreover, the Phase 3 results also showed that immune efficiency was much higher in those previously exposed to flaviviruses compared to those naïve (pooled vaccine efficacy of 78.2% and 38.1%, respectively)³⁷⁰, and a signal of increased risk of severe and hospitalized dengue in the 2-5 year age group during the longer-term hospital-based follow up³⁷¹. Because of these results, the younger age group was not included in the vaccine protocol and CYD-TDV was licensed in subjects between 9-45 years of age living in endemic countries³⁴².

5.4. Inactivated virus vaccines

Contrary to live attenuated vaccines, inactivated vaccines comprise the entire virion but without its capability to replicate; consequently, these vaccines tend to be more secure than the former because they don't have the possibility to revert their virulence, however, they tend to be weaker immunogens which is why they usually need the aid of adjuvants in order to induce an acceptable immune response³³⁶.

The first attempt to develop an inactivated vaccine against dengue dates back to works of Simmons, St. John and Reynolds in 1929²²⁰. After the standardization of adequate protocols for production and purification of the inactivated viruses, new candidates emerged: one of the most relevant efforts is a purified DENV 2 vaccine (DV2-PIV) developed by the Walter Reed Army Institute of Research (WRAIR) that has been shown to induce a protective immune response in non-human primates³⁷². The vaccine has been licensed to other developers who are currently testing several adjuvants to increase immunogenicity and evaluate different inactivating and purifying approaches; thus far, results on NHPs indicate that the tetravalent vaccine is able to induce a robust neutralising response against all DENV serotypes^{373,374}.

When compared to LAV vaccines, inactivated vaccines have the additional advantages of being able to stimulate both cellular and humoral adaptive immune response while not showing interference when several antigens are vaccinated together which, in the case of dengue, is important given that tetra-avalency is a requirement for the final formulation. However, they also supposed a much more complex production that usually leads to considerably higher costs per dose²²⁰.

5.5. Recombinant subunit vaccines

The advances made in molecular biology during the past twenty years have been applied to vaccine development efforts, with the idea of producing a viable and effective candidate using the right combination of epitopes in the form of viral proteins and peptides²²⁰. In this way, and contrary to the strategies described before, subunit vaccines target specific epitopes that are thought to be relevant for protection.

In the case of dengue, recombinant and subunit vaccines generally use recombinant truncated versions of E, or fractions of it (commonly DIII), as the main immunogenic component of the formulation²²⁰. A fundamental step in the production of these vaccines is obtaining the viral protein or peptide in an adequate concentration and purity; for this, several expression systems have been developed including yeasts, insect cells, bacteria, viruses (like baculovirus and vaccinia) and mammalian cells³⁷⁵. Although these vaccines have almost no safety- or reactogenicity-related issues, one major issue attached to this approach, is that incomplete or altered posttranslational processing of the antigen can lead to important differences between the antigen and the naïve protein, thus affecting the immune response obtain after vaccination²²⁰. In addition, purification of the antigen can be very demanding³⁴².

Available data on the candidates that have been tested so far indicate that using the E ectodomain as antigen generates good immunogenic responses and protection in the tested subjects, however further testing needs to be done to address tetravalent capacity³⁷⁵⁻³⁷⁷. Based on the differential immunogenic properties of E structural domains, several DIII-based subunit vaccines have been developed with different degrees of success³⁷⁸⁻³⁸⁵.

Besides the use of single, or partial, dengue proteins as vaccine antigens; these technologies have allowed also the design of fusion proteins in an attempt to increase the amount of neutralising epitopes available without increasing the risk of ADE. Some of these fusion candidates include DIII-Capsid (DIII-C)³⁸⁶, flagellin-E³⁸⁷ and tandem combinations of DIII from different serotypes^{388,389}.

DNA shuffling has also been employed to generate a recombinant E ectodomain³⁹⁰ or a consensus DIII antigen³⁹¹ capable of inducing a neutralising tetravalent response upon vaccination.

One of the major drawbacks of subunit vaccines has been limited immunogenicity, when compared to other technologies. To address low immunogenicity, subunit vaccines have used different adjuvant formulations without significant success³⁴². An alternative to the use of adjuvants is the reformulation of vaccine to display the antigen in an repetitive symmetrical structures that somewhat mimic the viral surface such as VLPs³⁴². In the case of dengue, several VLP-based vaccine candidates have shown enhanced immunogenicity in mice with on-going studies in NHPs^{392,393}.

5.6. Genetic vaccines

5.6.1. DNA vaccines

As a part of the new generation of vaccines, genetic vaccination offers the possibility to raise neutralising activity against a specific antigen without using whole viral particles or purifying the immunogen from exogenous expression systems²²⁰. These vaccines are

composed of a bacterial plasmid that serves as vector for the expression of a previously selected viral gene (or part of a gene) under the control of an efficient promoter and regulatory sequences according to the target cells; the plasmid is delivered into the host and internalised by cells that stimulate the immune response by producing and secreting the encoded protein³⁹⁴.

In this way, treated cells become antigen factories that are able to stimulate the full spectrum of the adaptive immune response: intracellular processed peptides are presented by the MCH molecules on the cell surface stimulating the cellular branch of the adaptive immunity while the secretion of the antigen by transfected cells is fundamental for the induction of specific antibodies. The stimulation of these two immunologic branches, which is highly desirable in the case of viral infections, is one of the most notable advantages of genetic vaccines³⁹⁵⁻³⁹⁷.

Since the protein is translated into the host's cells by its cellular machinery, the antigen goes through the same maturation process that the native viral protein encounters during the infection; in this aspect, genetic vaccination serves as a proper viral infection surrogate because the protein is expressed *in vivo*, which favours proper folding and post-translational modifications without the safety issues inherent to the administration of live viruses³⁹⁸⁻⁴⁰⁰. Additionally, genetic vaccines offer the advantage of very low cost production and excellent stability over a wide range of temperatures and periods of time, which can be critical when dealing with the conditions in some underdeveloped countries^{395,398}.

After the first clinical trial for a DNA vaccine⁴⁰¹, other candidates against various pathogens have been evaluated at Phase 1 levels⁴⁰²⁻⁴⁰⁶. In the case of dengue, most of the DNA vaccine candidates tested so far implicate the use of PrM-E, E ectodomain alone or a subunit form of E (mostly DIII) as antigens^{398,400,407,408}; likewise, attempts using non-structural proteins as antigens have also been reported^{305,409}.

The efforts of the US Naval Medical Research Centre (NMRC) have produced the most advanced DNA vaccine candidates and the only published a Phase 1 study using a plasmid expressing the PrM and E proteins of DENV1 (D1ME100)⁴¹⁰. Due to low immunogenicity, the vaccine was reformulated using a new adjuvant named Vaxfectin® that increased the immune response induced by tetravalent DNA vaccine in NHPs⁴¹¹. A Phase 1 study if this new formulation has been finished but the results are currently pending publication⁴¹².

In general, the studies involving DNA vaccines have showed that these type of formulations are well-tolerated and safe in humans⁴¹³ although low immunogenicity remains a main concern^{413,414}. A number of strategies have been implemented to enhance the immune response to DNA vaccines by improving DNA uptake, antigen expression and immune stimulation⁴¹², however, there's still no safe and effective DNA

vaccine available against dengue virus infection. Moreover, the unique concerns attached to this type of vaccination, which include the possibility of integration into the host genome and the eventual induction of anti-DNA antibodies that could lead to the development of autoimmune diseases²²⁰, have also made their implementation difficult.

5.6.2. Virus-vectored vaccines

One alternative to improve DNA or RNA delivery into the target cells is the use of viral vectors as vehicles, since infection-driven delivery of the genetic material is more efficient than transfecting cells *in vivo*⁴¹². Moreover, viral vectors are also able to induce direct stimulation of cytotoxic T cell responses³⁴².

In general, viral vectors are obtained from wild type viruses by deleting a part or all genes encoded in the genome⁴¹⁵. Immunogenicity of viral vectored-genetic vaccines is improved by the biological properties of the viral particle and can be design to target specific tissues of cell types⁴¹⁵. Due to genetic deficiencies, most viral vectors are not able to replicate and have proven to be safe, however, pre-existing antibodies against the vector may hamper the immunogenicity of the vaccine³⁴². In addition, the expression of remaining viral genes is still a concern as they could potentially influence the immune system⁴¹⁵.

In the case of dengue, adenovirus, poxvirus, measles and Venezuelan equine encephalitis virus (VEE), have been evaluated as potential platforms.

Adenoviruses have been extensively used as vectors for genetic vaccines. The development of a new generation of adenoviral vectors, capable of expressing multiple antigens, was used to develop a tetravalent dengue vaccine that was able to induce high neutralisation titres in NHPs^{416,417}. Nevertheless, due to increased risk observed in other candidates, the vaccine was later discontinued⁴¹².

Themis Bioscience together with Institut Pasteur, developed a tetravalent vaccine based on the expression of the DIII from all four serotypes in a live attenuated measles virus vaccine vector⁴¹⁸. The vaccine was able to induce neutralizing antibodies in mice and is being tested in NHPs³⁴².

VEE single-round infectious particles have been shown to express high level of recombinant antigens and efficiently infect dendritic cells³⁴². These properties, together with the low pre-existing immunity to VEE in humans, have added weight to their attraction as platforms for genetic vaccines⁴¹². However, the data regarding VEE-vectored dengue vaccines has been mixed: recently, a tetravalent candidate was shown to induce high level of neutralizing antibodies in mice and macaques^{419,420}, while other studies reported no significant difference in antibody titres or protection when comparing VEE-vectored vaccines against naked DNA immunisation⁴²¹.

Recently, Adeno-associated viruses (AAVs) have emerged as strong and convenient platforms for genetic vaccines. AAVs belong to the Parvoviridae family and are small, non-pathogenic, non-enveloped, single-stranded DNA viruses with a ≈ 4.7 kb genome composed of only two genes (rep and cap) and two flanking inverted terminal repeats (ITR)⁴²². As viral vector, replication deficient AAV are produced by replacing all viral coding sequences with the recombinant antigen while retaining the ITR regions⁴²³. This recombinant genome is then packaged into AAV particles by viral proteins given in *trans*, and purified from transfected cells. Contrary to other viral vectors, the more than 120 AAV serotypes and variants described are able to transduce a wide variety of tissues and provide alternatives to circumvent pre-existing immunity⁴²². However, low immunogenicity of AAVs when compared to other viral vectors, together with limited transgene capacity, high manufacturing cost and the need for high doses, are significant hurdles attached to AAV vaccine technology⁴¹⁵.

A vaccine candidate designed to express the E protein from all four dengue serotypes in a AAV-vectored system, demonstrated efficient antigen production and long-lasting neutralising responses in mice, although no further development has been reported since⁴²².

As emphasised throughout this introduction, the structure, functions and immunological properties of the different domains comprising the E protein of dengue virus, have been the subject of a significant amount of research during the last decades. Data regarding the neutralizing and cross-reactive capacity of antibodies directed against the E protein are mixed; nonetheless, there is a generalised tendency to emphasise both the complexity of the humoral immune response against dengue and the need to further examine the antigenic properties of E protein to develop an efficient and affordable vaccine.

In this context, this PhD research project had the objective of developing of a DNA vaccine against dengue virus using an integral approach that would incorporate a detailed biochemical analysis regarding the structural and functional properties of dengue virus E protein as the framework to study the immunogenic properties of designed vaccination constructs, formulations and strategies.

MATERIALS & METHODS

1. Cell lines

Mammalian cell lines HEK-293 (ATCC, Rockville, MD, USA, CRL-1573), HEK-293T/17 (HEK-293T, ATCC CRL-11268), Vero cells (ATCC CCL-81), U-2OS cells (ATCC HTB-96), HeLa cells (ATCC CCL-2) and HuH-7 (provided by Dr. Alessandro Marcello, ICGB, Trieste, Italy) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Life Technologies), 50 µg/ml gentamycin and 2 mM L-glutamine. In addition, Vero FM cells (Vero E6 derivative used for viral amplification, provided by Dr. Toni Rieger, BNI, Hamburg, Germany) were maintained in the same conditions with 1% non-essential amino acids. K562 cells (ATCC CCL-243) and THP-1 human monocytic cells (ATCC TIB-202), were maintained in Roswell Park Memorial Institute (RPMI) 1640 Medium (Life Technologies) supplemented with 10% FCS and 50 µg/ml gentamycin. To select HEK-293 stable clones, 0.4 mg/ml geneticin (G418, Life Technologies) was added to the culture medium. Unless indicated differently, all cell cultures were grown and maintained at 37°C with 5% of CO₂.

2. Viruses

DENV1 Hawaii A strain, DENV2 NGC strain, DENV3 3140/09 isolate, DENV4 TC25 strain, ZIKV 976 Uganda strain, WNV New York 1999 strain and YFV French neurotropic virus strain (provided by Dr. Alessandro Marcello, ICGB, Trieste, Italy) were used. All viruses were propagated in Vero cells using DMEM medium containing 2% heat inactivated FCS (DENV3 that was propagated in Vero FM cells). After amplification, viral stocks were prepared to a final FCS concentration of 20-30% and kept at -80°C until used. Viral titres were determined by plaque assay (PFU/ml) or foci forming assay (FFU/ml) on Vero cells.

3. Plasmid DNA constructs

Sequences coding for PrM and E ectodomain (sE) were obtained for DENV1 Nauru Island strain (GenBank accession number U88535.1), DENV2 New Guinea C strain (GenBank accession number AF038403), DENV3 3H87 strain (GenBank accession number M93130), DENV4 Dominica strain (GenBank accession numbers AF326573.1) and ZIKV Mr766 strain (GenBank accession number AEN75266.1).

Codon-optimised sequences for mammalian expression of PrMsE from DENV2, DENV3 and ZIKV, and sE from DENV1 and DENV4, were obtained as synthetic fragments in pUC57 vectors (GenScript, Piscataway, NJ, USA). Synthetic fragments carrying the viral-encoded and codon-optimised DIII sequences of all DENV serotypes were also obtained (E protein codons 297-416 for DENV1, DENV2 and DENV4; and 295-414 for DENV3). Each PrMsE, sE and DIII (optimised and non-optimised) sequence were

cloned in pcDNA3.1 vectors (Life Technologies) and fused to an immunoglobulin leader sequence (sec) at the N-terminus⁴²⁴ and to the SV5 tag (GKPIPPLLGLD)⁴²⁵ at the C-terminus, to obtain PrMsE-SV5, sE-SV5 and DIII-SV5 constructs, respectively. DIII- γ constructs (carrying either the optimised or non-optimised DIII sequences) contained, in addition, the human IgG heavy chain constant domain 3 (γ CH3) downstream of the SV5 tag. MsE-SV5 and sE-SV5 constructs of DENV2, DENV3 and ZIKV were obtained after deleting the Pr or PrM coding regions, respectively; while DI/DII-SV5 constructs (E protein codons 1-296 for DENV1, DENV2 and DENV4; and 1-294 for DENV3) resulted from removing the DIII coding regions from DENV-derived sE-SV5 plasmids. Regarding chimeric constructs, sE(DIII) chimeras were obtained by substituting the DI/DII sequence with that of another serotype, while DI-DII chimeras were obtained as synthetic fragments and then cloned either into the same pcDNA3.1 vectors to obtain DI-DII-SV5 chimeras, or into sE constructs (to obtain sE(DI) and sE(DII) chimeras). sE-SV5, DI/DII-SV5 and optimised DIII- γ constructs were cloned into pVAX1 vectors (Life Technologies) as well.

For constructs requiring particular aminoacid mutations, site-directed mutagenesis (QuikChange XL Site-Directed Mutagenesis Kit, Agilent Technologies, La Jolla, CA, USA) was performed following the instructions of the manufacturer. This was used for:

1. Non-glycosylated mutants (Asn67Gln-Asn153Gln) of DENV3-derived DI/DII-SV5 and sE-SV5 plasmids.
2. cvD variants (Covalent E dimers) of PrMsE, MsE and sE constructs from all four DENV serotypes and ZIKV (Ala259Cys for DENV1, 2 and 4, Ala257Cys for DENV3 and Ala264Cys for ZIKV).
3. To obtain the DIII- γ construct from DENV4 TC25 strain that differs from the Dominica strain in three aminoacids (Leu357Phe, Asn360Tyr and Asn384Asp).

For experiments involving biotinylated proteins, coding sequences were cloned into a bigenic pcDNA3.1 vector containing the BAP tag (biotin acceptor peptide, GLNDIFEAQKIEWHE)^{426,427} followed by roTag⁴²⁸ at the C-terminal end of the encoded protein (BAP-roTag), and the gene for a secretory *Escherichia coli* biotin ligase (sec-BirA)⁴²⁶. Likewise, the DIII- ϵ CH4 constructs were obtained by fusing DIII sequences to the human ϵ CH4 followed by the BAP tag in a modified version of the bigenic vector⁴²⁹.

Constructs targeted for membrane display were engineered from SV5-tagged plasmids by replacing the SV5 tag, with the transmembrane and cytoplasmic domains of the human MHC-I α chain.

For production of pseudoviruses, codon optimised sequences coding for *C-PrM-E* genes of ZIKV Mr766 strain and a chimera containing the C gene from ZIKV Mr766 and the *PrM-E* from DENV2 New Guinea C strain (C^Z -PrME^{D2}), were obtained as synthetic fragments and cloned into pVAX1 vectors. Plasmids expressing only the capsid protein

(C) from ZIKV or PrME from DENV2 were obtained by deleting the PrME and C coding regions from the chimeric constructs, respectively.

4. Production of recombinant Adeno-Associated Virus vectors

Codon optimised DIII sequences from DENV3 fused to the sec signal at the N-terminus and the γ CH3 domain downstream of the SV5 tagged at the C-terminus, were cloned into pAAV-MCS vector (AAV Helper-Free System, 240071, Agilent Technologies).

Recombinant AAV vectors used in this study were prepared by the AAV Vector Unit at the International Centre for Genetic Engineering and Biotechnology Trieste, as previously described⁴³⁰. Briefly, infectious recombinant AAV vector particles were generated in HEK-293T cells culture in roller bottles by a cross-packaging approach whereby the vector genome was packaged into AAV capsid serotype-9⁴³¹.

Viral stocks were purified by PEG precipitation from clarified cell lysates and cell culture supernatant followed by two consecutive CsCl₂ gradient centrifugations⁴³².

Full viral particles obtained from the gradient were extensively dialyzed in Phosphate buffer solution (PBS) and stored in aliquots at -80°C until use.

The physical titre of recombinant AAVs was determined by quantifying vector genomes (vg) packaged into viral particles, by real-time PCR against a standard curve of a plasmid containing the vector genome; values obtained were in the range of 1×10^{12} to 1×10^{13} vg/ml.

5. Animal Immunisations

Different groups of 5-6 weeks old, female Balb/c mice (purchased from Harlan, Milan, Italy) were used for vaccination throughout the project. Since the groups differed significantly depending on the experiment, the number of animals included in each group is mentioned in the main text.

For DNA immunisations, mice were immunised intradermally using Gene Gun technology (Bio-Rad, Hercules, CA, USA). Before vaccination, the abdominal area of each mouse was shaved and 1 μ m gold particles coated with ≈ 1 μ g of plasmid DNA were shot at 400 psi. Unless indicated otherwise, mice were immunised three times at two weeks intervals (Days -30, -15 and 0). In the case of the DIII-tetravalent formulation, animals followed the same immunisation protocol, but were vaccinated with two-1 μ g shots of DNA each time.

For AAV-vectored immunisations, 100 μ l doses of 3×10^{10} viruses were administered either by intramuscular injection in the posterior aspect of the hindlimb, or subcutaneously depending on the vaccination protocol followed.

Blood samples were collected by sub-mandibular puncture at least 15 days after vaccination. However, bleeding times varied significantly depending on the experiment

and are thus detailed when needed. Serum was separated from the blood samples, cleared by centrifugation and stored at -20°C until use.

All animal procedures were approved by the Italian Ministry of Health (Ministero della Salute) and the ICGEB Animal Welfare Board (protocol DGSAF0024706) in compliance to laws and policies established in the legislation D. L.vo 26/2014 of the Italian Government.

6. Monoclonal antibodies (mAb)

Purified human mAbs 2D22, 1F4 and 5J7 as well as mouse mAb 4G2, were kindly provided by Prof. Aravinda de Silva (University of North Carolina, Chapel Hill, NC, USA). Mouse mAb 4G2 was also provided by Dr. Vivian Huerta (Centre for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba) and purchased from Millipore (clone lone D1-4G2-4-15, MAB10216, Millipore, Temecula, CA, USA) as well. Mouse mAb dengue 1-11 was purchased from AbD Serotec (Now Bio-Rad) (clone dengue 1-11(3), MCA227, Bio-Rad). Mouse mAb 4E5A was kindly provided by Prof. Jonathan R. Lai (Albert Einstein College of Medicine, NY, USA). mAbs EDE1-C8 (clone 752-2 C8), EDE1-C10 (clone 753(3) C10) and EDE2-B7 (clone 747 B7) were constructed as human IgG antibodies from previously deposited sequences²⁸⁴ and used as culture supernatants from transfected cells.

7. Expression of recombinant proteins

Transient transfections of HEK-293T cells, and stable transfections of HEK-293 cells were performed as described by Sambrook *et al.*⁴³³, using circular or linearized plasmids, respectively. Transfections of HeLa, U-2OS and Vero cells were performed using Lipofectamine 3000 (Life Technologies) according to manufacturer's instructions.

Cells were seeded in 6-well plates at 5×10^5 cells/well density 24h before transfection. Depending on the experiment, transient transfections were done using standard calcium phosphate method with 0,5-3 µg of plasmid DNA. After overnight incubation at 37°C, culture medium was replaced with serum-free medium and cells incubated for another 24h at 37°C (or 28°C, as indicated). Serum-free media was supplemented with 100 µM biotin when required (Sigma-Aldrich, St. Louis, MO, USA, B4501). Culture supernatants were collected and cleared by centrifugation (10,000rpm for 10min), while total cellular extracts were prepared in 100 µl of TNN lysis buffer (100 mM Tris-HCl, pH 8, 250 mM NaCl, 0.5% NP-40) supplemented with Protease Inhibitor Cocktail (PIC, Sigma, P8340) at 4°C. If needed, free biotin was removed from cultures supernatants by dialysis against PBS using Spectra/Por® 4 dialysis membranes (MWCO:12-14 kDa, Spectrum Labs, CA, USA). For hetero-dimerisation assays, SV5-tagged and BAP-tagged (or membrane-displayed) constructs, were co-transfected in a 2:1 ratio. All samples were kept at -20°C until use.

Where indicated, cell extracts and culture supernatants samples were treated with Peptide-N-Glycosidase-F (PGNase F, New England Biolabs, P0704, Ipswich, MA, USA).

To produce large amounts of biotinylated antigens for ELISA, the transfection procedure was scaled up to 10cm or 15cm culture plates. Alternatively, recombinant biotinylated DIII- ϵ CH4-BAP of all four DENV serotypes, sE-BAP-roTag proteins of DENV3 and DENV4, were expressed in stably-transfected HEK-293 cells. Stable transfection was done with 15 μ g of BglII (New England Biolabs, R0144) -linearized DNA using calcium phosphate technique. Secretion of biotinylated proteins from stably-transfected HEK-293 clones was screened by ELISA and further confirmed by western blot. Supernatants from selected clones were collected after 72 h of culture in serum-free medium supplemented with biotin and dialyzed as before. In experiments involving the use of denatured biotinylated DIII- ϵ CH4-BAP antigens, the dialyzed supernatants were denatured in presence of 0.5% SDS (Sigma-Aldrich) and 2.5% β -mercaptoethanol (Sigma-Aldrich) and boiled for 10min. N-Ethylmaleimide (NEM, Sigma-Aldrich) was then added and samples were extensively dialyzed against PBS before using.

8. Western blot (WB) and Slot blot (SB) analyses.

Samples were separated by reducing or non-reducing 10%, 12% or 4-12% gradient SDS-PAGE. When needed, samples were denatured by supplementing the loading buffer (25 mM Tris-HCl pH 6.8, 1% SDS, 10% glycerol) with 175 mM β -mercaptoethanol, and boiling for 10min.

After electrophoresis, samples were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore) and blocked with a 5% milk solution in PBS (PBS-milk). PBS and PBST buffer (0.05% Tween 20 in PBS, pH 7.4) were used for washing. When detecting SV5-tagged proteins, membranes were incubated for 1h with anti-SV5 mAb (1 μ g/ml in PBS-milk), washed, and probed with HRP-conjugated anti-mouse IgG goat antibodies (KPL, Gaithersburg, MA, USA, 074-1809, 1:10,000 in PBS-milk) for 1h. In the case of biotinylated proteins, membranes were probed with HRP-linked streptavidin (Jackson ImmunoResearch, Newmarket, UK, 016-030-084, 1:20,000 in PBST) for 1h after blocking. Mouse anti-tubulin mAb (clone DM1A, Millipore, MABT205, 1:5,000 in PBS-milk) or mouse HRP-conjugated anti-actin mAb (clone AC-15, Sigma-Aldrich, A5441, 1:30,000 in PBS-milk) were used as loading controls. Signals were developed by ECL (ThermoFisher-Pierce, Rockford, IL, USA). Unless otherwise indicated, equivalent amounts of cellular extracts and culture supernatant samples were used in each experiment (0.5% of total sample).

For Slot Blots, normalised samples were prepared in native (125 mM Tris-HCl pH 6.4) or denatured (125 mM Tris-HCl pH 6.4, 175 mM β -mercaptoethanol) loading buffers, and blotted onto PVDF membranes using the Bio-Dot SF Apparatus (Bio-Rad, Hercules, CA,

USA). In addition, denatured samples were boiled for 10min before blotting. Incubation with anti-SV5 (1 µg/ml in PBS-milk) or 4G2 (50 ng/µl in PBS-milk) was followed by and HRP-conjugated goat anti-mouse IgG (1:50,000 in PBS-milk). As before, signals were developed by ECL.

When needed, bands were quantified using ImageJ 1.47v software (National Institute of Health, USA) or the Alliance detection system (UVItec, Cambridge, UK).

9. Immunoprecipitations

HEK-293T cells were transfected with different combination of DNA plasmids encoding sE or sE-derived constructs. Culture supernatants and cell lysates were prepared as before. Samples (diluted in PBS if needed) were incubated with anti-SV5 (1 µg/ml in TNN) and protein A agarose (Repligen, Waltham, MA, USA) for 2h at 4°C. Samples were then loaded onto Micro Bio-spin columns (Bio-Rad) and washed with TNN buffer, followed by RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 1 mM EDTA). Immunoprecipitated proteins were eluted with from the agarose beads with 50 µl of reducing loading buffer, and detected on SDS-PAGE following the WB procedure. In the specific case of co-immunoprecipitated biotinylated proteins, samples were detected with HRP-conjugated streptavidin. The relative amount of immunoprecipitated sample loaded is indicated when needed.

10. Radioactive Labelling

Transfected HEK-293T cells were incubated for 30min in L-methionine and L-cysteine free DMEM (Life Technologies) supplemented with dialyzed FCS. Cells were then labelled with 200 µCi/ml of a [³⁵S]-methionine and [³⁵S]-cysteine mix (Expre³⁵S, Perkin Elmer, Waltham, MA, USA) in the same methionine and cysteine-free medium, and incubated for 15min. Cells were then collected and lysed in TNN buffer with PIC. Samples were immunoprecipitated with anti-SV5 antibody and protein A-agarose as previously described. Immunoprecipitated proteins were eluted from agarose beads and separated on 10% SDS-PAGE. After electrophoresis, gels were fixed (using a 10% acetic acid, 10% methanol solution), incubated for 20min in Amplify fluorographic enhancer (GE Healthcare, Little Chalfont, UK), dried and exposed to autoradiography on Kodak BioMax XAR films (Carestream Health, Rochester, NY, USA).

11. ELISA

Mouse sera, culture supernatants and different purified mAbs were tested on mono-biotinylated DIII-εCH4-BAP, sE-BAP-roTag, DI/DII-BAP-roTag and sE-cvD-BAP-roTag proteins. The relative concentrations of biotinylated proteins collected from transiently transfected HEK-293T and the stably transfected HEK-293 clones were normalized by

WB with HRP-conjugated streptavidin on the Alliance detection system, and comparable amounts of biotinylated protein were used for coating the ELISA plates.

Nunc Maxi Sorp Immuno-Plates (ThermoFisher-Nunc, Roskilde, Denmark) were pre-coated with 100 μ l/well of 5 μ g/ml avidin (Sigma) in 50 mM Na₂CO₃/NaHCO₃ buffer (pH 9.5) and incubated overnight at 4°C. Plates were washed in PBST buffer (0.05% Tween 20 in PBS pH 7.4), blocked with 1% Bovine serum albumin in PBST (PBST-BSA) for 1.5h at room temperature (RT), and second-coated with the dialyzed biotinylated-antigen diluted in PBS (100 μ l/well) at 4°C overnight. Normalisation of captured DIII- ϵ CH4 biotinylated proteins was further confirmed by ELISA using HRP-conjugated anti-human IgE goat antibodies (KPL, Gaithersburg, MA, USA, 074-1004, and 1:5,000 in PBST-BSA). For sE-derived proteins from the same DENV serotype, normalisation was confirmed also by ELISA, using mAb 4G2 (0.15 ng/ μ l in PBST-BSA) and HRP-conjugated anti-mouse IgG γ -chain goat antibodies (Jackson ImmunoResearch, code 115-035-071, 1:50,000 in PBST-BSA) instead^a.

For assays designed to evaluate the structural features of captured biotinylated proteins, plates were incubated for 1h with 100 μ l/well of different mAbs diluted in PBST-BSA (mAb 4G2, 0.15 ng/ μ l; mAb 4E5A, 0.5 ng/ μ l; mAb 2D22, 0.5 ng/ μ l; mAb 1F4, 0.5 ng/ μ l; mAb 5J7, 0.5 ng/ μ l; mAbs EDE1-C8, EDE1-C10 and EDE2-B7 were used as undiluted supernatants) after washing. The plates were then washed and, depending of the mAb used, 100 μ l/well of HRP-linked anti-mouse IgG γ -chain goat antibodies (1:50,000 in PBST-BSA) or HRP-conjugated anti-human IgG goat antibodies (KPL, 074-1002, 1:20,000 in PBST-BSA) were added and incubated for 1h at RT. The bound conjugate was detected using TMB substrate (Sigma), stopped with H₂SO₄ 1M and measured at 450 nm (OD₄₅₀) on a Bio-Rad iMark microplate reader.

For analyses involving sera of vaccinated animals, 100 μ l of different sample dilutions (in PBST-BSA) were added to antigen coated plates and incubated for 2h at RT. After washing, 100 μ l/well of HRP-linked goat antibodies anti-mouse IgG γ -chain (1:50,000 in PBST-BSA) were added and incubated for 1h at RT. The bound conjugate was detected as before. The anti-dengue IgG titres were determined as the reciprocal of the dilution at which the OD₄₅₀ was 3 times higher than that of the negative control serum. Negative control sera obtained from non-vaccinated animals (pre-immune sera) showed the same performance as sera from animals immunised with a construct containing an irrelevant protein fused to γ CH3 or sera from animals immunised with empty vectors.

^a Given the different reactivity of mAb 4G2 towards the FL of the four DENV serotypes and ZIKV, normalisation of the amount of protein captured in ELISA was restricted to constructs of the same origin.

11.1. Evaluation of anti-DIII specific antibody concentration

Approximate concentrations of anti-DIII specific antibodies in immunised mice were determined by interpolating the OD₄₅₀ from different sera dilutions on its homologous DIII antigen, in a calibration curve obtained by plotting the OD₄₅₀ values from an ELISA on biotinylated 3sE with different amounts of a purified and previously quantified sample of mAb 4G2^b. The concentrations are reported as arithmetic means ± standard deviations of the values obtained for all the dilutions tested.

11.2. Dengue virus-capture ELISA

Plates were coated with 100 µl of a purified anti-DENV immunoglobulin fraction from a human serum cross-reactive with all 4 serotypes (provided by Dr. Vivian Huerta, Centre for Genetic Engineering and Biotechnology (CIGB), Habana, Cuba) (15 mg/ml in 50mM Na₂CO₃/NaHCO₃ buffer, pH 9.5) and incubated overnight at 4°C. Plates were then washed with PBS, blocked with 1% BSA in PBS (PBS-BSA), and second-coated with 4x10⁴ PFUs/well of each viral serotype for 2h at RT. After washing, plates were incubated for 1h at 36°C with 100 µl/well of the different anti-DIII sera (diluted to an approximate concentration of 100 ng/ml in PBS-BSA) or negative control sera at an equivalent dilution. mAb dengue 1-11, specific for DENV1 envelope protein (1 µg/ml in PBS-BSA) and a dilution of a DENV panreactive mouse serum (also provided by Dr. Vivian Huerta, Centre for Genetic Engineering and Biotechnology (CIGB), Habana, Cuba) were used as positive controls. For detection, HRP-conjugated goat anti-mouse IgG γ-chain (1:50,000 in PBS-BSA) was used.

11.3. Avidity assay

Serum avidity indexes were measured by a modified ELISA protocol with urea washes^{434,435}. Briefly, sera samples were tested at different dilutions corresponding to an OD₄₅₀ value of 0.5-0.9. After incubation with serum, plates were washed two times (3min each) with 200 µl of PBST, with or without 6M urea, and incubated with secondary antibody as described above. The avidity index was calculated using the following formula:

$$\text{avidity index (\%)} = \frac{OD_{450} \text{ with Urea}}{OD_{450} \text{ without Urea}} \times 100$$

^b In our case, the antibody reacted strongly against the 3sE protein with almost 100% avidity, and was therefore used to generate the calibration curve.

12. Cytofluorimetry

HEK-293T cells were transfected with DNA plasmids encoding different sE or sE-derived constructs as described before. Cells were then washed with PBS and resuspended in PBS-BSA (2%) supplemented with 5 μ M EDTA. Samples were then incubated with different mAbs (anti-SV5, 1 ng/ μ l; mAb 4G2, 1 ng/ μ l; mAb 4E5A, 1 ng/ μ l; mAb 2D22, 1 ng/ μ l; mAb 1F4, 1 ng/ μ l; mAb 5J7, 1 ng/ μ l; mAbs EDE1-C8, EDE1-C10 and EDE2-B7 were used as undiluted supernatants) or anti-3sE pooled sera from immunised animals (1:500 dilution), in PBS-BSA, for 1h at RT followed by Alexa488-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, 115-545-062, 1:1,000 in PBS-BSA) or Alexa488-conjugated goat anti-human IgG (Jackson ImmunoResearch, 109-545-003, 1:1,000 in PBS-BSA), and analysed for relative alexa-488 fluorescence in a FACSCalibur (BD Biosciences, San Jose, CA, USA). Cytofluorimetry data were analysed using FlowJo 7.6.5 software (LLC, Ashland. OR, USA).

13. Immunofluorescence

Vero cells were seeded in sterile cover glasses 24h before the assay and infected with virus preparations at a multiplicity of infection (MOI) of 0.1 for 36h (24 h when using WNV). Cells were then fixed with 3.7% paraformaldehyde (PFA, Sigma-Aldrich) in PBS for 20min and quenched with 150 mM glycine in PBS (two washes of 10min at RT). After washing with PBS, cells were permeabilised with 1% Triton (Sigma-Aldrich) in PBS for 15min, and blocked with PBST-BSA (0.1%) for 1h. Viruses in infected cells were detected using different anti-DIII, anti-DI/DII, anti-sE sera or pre-immune control sera (at 1:100 dilution in PBS-BSA), or mAb 4G2 (50 ng/ μ l in PBS-BSA) followed by Alexa488-conjugated goat anti-mouse IgG (1:1,000 in PBS-BSA). Labelled cells were mounted with ProLong supplemented with DAPI (Thermo Fisher Scientific). Images were acquired using a Zeiss LSM510 META microscope or, alternatively, a Nikon Eclipse Ti-E microscope.

Transfected HEK-293T cells were treated as described for the cytofluorimetry assays and then plated in glass slides previously treated with poly-lysine (Sigma-Aldrich) to promote attachment. After 1h incubation at RT, cells were fixed with 3.7% PFA in PBS for 10min and quenched with 150 mM glycine in PBS as before. For permeabilisation, cells were treated with 1% Triton (Sigma-Aldrich) in PBS for 10min. Samples were then incubated with anti-SV5 (1 μ g/ml in PBS-BSA) for 1h at RT, followed by Alexa488-conjugated goat antibodies anti-mouse IgG (1:1000 in PBS-BSA). As before, cover glasses were mounted with DAPI-supplemented ProLong and images were acquired using a Zeiss LSM510 confocal microscope.

14. Production and analysis of Pseudoviruses

Pseudoviral particles were produced by co-transfection of a DNA-launched WNV replicon expressing EGFP⁴³⁶ (WNV-rep, provided by Theodore Pierson, National Institute of Allergies and Infectious Diseases, MD, USA) with plasmids expressing the structural genes of ZIKV or DENV2 in *trans*, as previously described⁴³⁷. Briefly, HEK-293T cells were transfected with 2 µg of DNA (1:3 ratio between WNV replicon and packaging plasmids, respectively) using linear polyethylenimine (1 µg/µl in H₂O, pH 7.4; used at a 6:1 ratio with the amount DNA transfected) (PEI, MW 25,000, Polysciences, 23966, Warrington, PA, USA). 16h post-transfection, culture medium was replaced with a DMEM supplemented with 7% FCS and incubated for another 48h at 37°C or 28°C. Pseudoviruses were harvested in cell culture supernatants, clarified from cell debris by centrifugation (10min at 10,000 rpm) and stored at -20°C.

14.1. Detection of pseudoviral particles in WB

Pseudoviral preparations were concentrated by ultracentrifugation at 40,000g for 24h. Afterwards supernatants were discarded and pellets were resuspended in WB loading buffer. Samples were then separated by non-reducing 4-12% gradient SDS-PAGE, transferred to PVDF membranes and blocked as before. For detection of pseudoviruses, membranes were incubated with mAb 4G2 (50 ng/µl in PBS-milk) for 1h, washed, and treated with HRP-conjugated anti-mouse IgG goat antibodies. Signals were developed by ECL.

14.2. Quantification of pseudoviruses by Real-Time PCR.

WNV Replicon-derived RNA was isolated from 100 µl of pseudoviral preparations using the RNAzol-BEE solution (Tel-Test, Friendswood, TX, USA) following manufacturer's instructions. For cDNA synthesis, samples were first treated with DNaseI (Promega, Madison, WI, USA), and reverse transcription was performed using random hexamers (Sigma) and M-MLV Reverse transcriptase (Life Technologies) according to the manufacturer's protocols. cDNAs were used as templates for quantitative real-time PCR using WNV-3'UTR specific primers (Forward, 5'-CAGTGTCAGACCACACTTTAATGT-3'; Reverse, 5'-GCTTACAGCTTCAGCCAAG-3') and EvaGreen Technology (Bio-Rad, Hercules, CA, USA) on a C1000 thermal cycler using CFX96™ Real-Time Detection System (Bio-Rad) following manufacturer's instructions (Melting temperature: 67°C). Quantification of the relative number of viral RNA genomic copies was calculated by interpolating Cq values for each sample (done in duplicate) in a standard curve made with a quantified WNV-rep plasmid ($r^2=0.9972$). Values outside the range covered by the standard curve were considered negative.

14.3. Immunofluorescence.

Vero cells (seeded as mentioned before) were infected with different pseudoviral preparation using a 0.1 MOI. 48 h post-infection cells were fixed with 3.7% PFA in PBS for 20min and quenched with 150 mM glycine in PBS. After washing, cells were mounted with ProLong supplemented with DAPI. Images were acquired using a Nikon Eclipse Ti-E microscope.

14.4. Infection of mammalian cells.

24h before infection, cells were seeded at a density of 4×10^4 cells/well in 24-well plates. Cells were infected with 2×10^4 pseudoviral particles diluted to 200 μ l in serum-free DMEM for 3h at 37°C. 0.5 ml of DMEM with 2% FBS were then added and cells were further incubated for 48h. After incubation, cells were resuspended, counted and analysed for EGFP expression by cytofluorimetry as above.

14.5. Antibody-dependent pseudoviral infection of K562 cells.

Equal volumes of 1:100 dilutions of either anti-4DI/DII or pre-immune sera, and different preparations of pseudoviral particles containing with 2×10^4 pseudoviral particles, were mixed and incubated for 1.5h at 37°C in round-bottom 96 multi-well plates (Corning-Costar, Corning, NY, USA). 5×10^4 K562 cells were added to the serum-virus mixture to a final volume of 200 μ l, and incubated for 48h at 37°C. Cells were then washed, resuspended in PBS, counted and analysed for EGFP expression by cytofluorimetry as above.

15. Neutralisation assays

15.1. Plaque reduction neutralisation assay (PRNT₅₀).

PRNT were performed on Vero cells seeded at a density of 1.6×10^4 cells/ well in 24 multi-well plates 24h before infection. Sera samples from vaccinated mice were de-complemented (30min at 56°C) and serially two-fold diluted in serum-free DMEM medium. Serum dilutions were mixed with equal volumes dengue virus preparations containing 50 PFUs in serum-free DMEM medium, and incubated for 1.5h at 36°C. Vero cells were first washed, infected with the virus-serum mixture and further incubated for 1h at 36°C. Afterwards, the viral inoculum was removed and cells were overlaid with 1ml of DMEM with 2% FCS and 3% carboxymethylcellulose (CMC, Sigma). Plates were incubated at 36°C for 7-8 days depending on serotype (7 days for DENV2 and DENV3, 8 days for DENV1 and DENV4). After this period, cells were washed twice with PBS, fixed for 20min with PFA 3.7% and stained with 1% crystal violet for 30min. Plaques were counted and percentage of plaque reduction against control serum was calculated as follows:

$$\% \text{ of plaque reduction} = 100 - \left(\frac{100 \times \overline{\text{plaques}}_{\text{sample}}}{\overline{\text{plaques}}_{\text{pre-immune sera}}} \right)$$

Neutralising antibody titres were expressed as the serum dilution yielding a plaque reduction of 50% (PRNT₅₀).

15.2. Foci reduction neutralisation test (FRNT₅₀).

FRNT were carried out in Vero cells, seeded at a density of 6.5x10⁴ cells/well in 48 multi-well plates, 24h before infection. Preparation of sera samples, viral inoculum and cell infection were performed as described for PRNT₅₀. After 3 days incubation at 36°C (48h for WNV), cells were washed and fixed for 20min with PFA 3.7%, permeabilised with 1% Triton in PBS for 10min and treated with 0.3% H₂O₂ solution in methanol for 30min. Infection foci were developed by after incubation with mAb 4G2 (1 ng/μl in PBS-BSA) for 1h at room temperature (RT), followed by incubation with HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, 1:1,000 in PBS-BSA). Foci were then stained with 100 μl True-Blue reagent (KPL, Gaithersburg, MA, USA) and counted. Following the calculation done for PRNT₅₀, neutralising antibody titres were expressed as the serum dilution yielding a 50% foci reduction when compared against pre-immune sera (FRNT₅₀).

16. Antibody-dependent enhancement of infection assays

16.1. ADE in THP-1 cells.

This assay was performed by researchers at the Ooi Lab. (Emerging Infectious Diseases Programme at the Duke-NUS Medical School in Singapore) using a previously described ADE method⁴³⁸. Briefly, serial two-fold dilutions of each serotype-specific anti-DIII pooled sera were incubated with the virus for 1h at 37°C before being added to THP-1 cells at a MOI of 10. 72h post-infection, the culture was clarified by centrifugation, and the infectious titre of dengue virus in the culture supernatant was quantified by plaque assay.

16.2. ADE in K562 cells.

10-fold serum dilutions were mixed with an equal volume of RPMI 1640 serum-free medium containing 4x10³ FFUs of each virus and incubated for 1.5h at 36°C in round-bottom 96 multi-well plates. K562 cells were added to the serum-virus mixture to achieve a final MOI of 0.1, and incubated for 72h (48h for WNV) at 36°C. Cells were then fixed for 30min with PFA 2% on ice, and blocked in permeabilisation buffer (0.1% saponin (Sigma-Aldrich), 2% FCS and 0.1% NaN₃ in PBS) for 30min at 4°C. After blocking, cells were incubated with mAb 4G2 (1 ng/μl in permeabilisation buffer) followed by Alexa 488-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, 1:1000 in permeabilisation

buffer), both for 1h at RT. After washing, cells were resuspended in PBS containing 2% FCS and 0.1% NaN₃, and analysed by cytofluorimetry as before.

17. Statistical analysis.

Experimental data presented in this project were obtained from at least three independent experiments done in duplicate or triplicate. Unless indicated otherwise, arithmetic means \pm standard deviations were calculated. Unpaired two-tailed *t* test, and One-way ANOVA were used for analyses when needed (GraphPad Prism 6.0, GraphPad Software Inc., La Jolla, CA, USA); in all cases *p* values <0.05 were considered significant and variances between compared groups were not significantly different, when required. Sample size was not statistically assessed and data distribution was assumed to be normal. No randomization was done. Data collection or analysis was not blinded.

RESULTS & DISCUSSION

PART I

ANALYSIS OF E PROTEIN PRODUCTION, FOLDING, DIMERISATION AND SECRETION FROM MAMMALIAN CELLS

1.1. SYNOPSIS

To obtain a secretory version of E, the stem and anchor regions at the C-terminal end of the protein are usually removed. The resulting protein, known as E ectodomain, or soluble E (sE), is composed of the three main structural domains: DI, DII and DIII and, as mentioned before, is the main antigen for most of the described DENV vaccines based on subunit and genetic approaches. With the objective of characterising the secretory properties of the E ectodomain, we performed an exhaustive biochemical study of the production, folding and secretion of sE constructs from all 4 DENV serotypes and ZIKV in mammalian cells. Our data indicate that DII is fundamental for the secretion of sE, while DIII serves a pivotal function in stabilisation of the dimeric conformation of E.

In addition, and given the importance of complex quaternary epitopes for inducing a strong neutralising response against flaviviruses, we also studied the dynamics involved in E dimerisation and demonstrate that this arrangement, and the overall folding of sE, is independent of PrM but highly dependent on temperature.

Finally, we also show that the efficient antigen secretion from transfected cells determines the strength of the antibody response elicited following DNA vaccination, an aspect that should be seriously considered when developing DNA vaccines.

1.2. RESULTS

As a disclaimer, the reader is informed that some of the results included in this section were previously published in *The Journal of General Virology*⁴³⁹, while an additional research article including most of the remaining data was recently submitted.

1.2.1. DII determines sE secretion from mammalian cells.

The secretory profiles of recombinant sE from all DENV serotypes were investigated by transfecting mammalian cells with constructs encoding truncated versions of E where the stem-anchor regions were substituted with an SV5 tag at the C-terminus. To ensure proper translocation to the ER upon translation, the wild type leader peptide signal from the different proteins was substituted with a genomic fragment of a mouse signal secretion peptide from an immunoglobulin V_H gene (sec) at the N-terminus⁴²⁴, this signal has been shown to be efficiently cleaved by the ER signal peptidase within the mammalian ER in a co-translational manner⁴²⁴, which allowed us to circumvent the variability derived from differences between the secretory signals of different proteins and viruses used throughout the project. Schematic representations of these and other constructs used in the experiments described in this section are shown in Fig. 21a. sE secretion was assessed by western blot (WB) of cellular extracts and culture supernatants of transfected HEK-293T cells. The presence of the protein in culture supernatants indicated an efficient secretory phenotype; conversely, a poor-secretory phenotype was assigned to the proteins that were mainly retained within transfected cells with only traces found in the supernatants. As shown in Fig. 21b, significant secretory phenotype differences were observed among the four DENV serotypes: while sE constructs from DENV 3 and DENV4 (3sE and 4sE, respectively) were efficiently secreted from transfected cells, proteins derived from DENV1 and DENV2 (1sE and 2sE, respectively) showed a poor-secretory phenotype since they were mainly retained intracellularly (Fig. 21b, long exposure). In addition, the secretory behaviour of the sE constructs was independent of the mammalian cell line used for transfection (Fig. 21c).

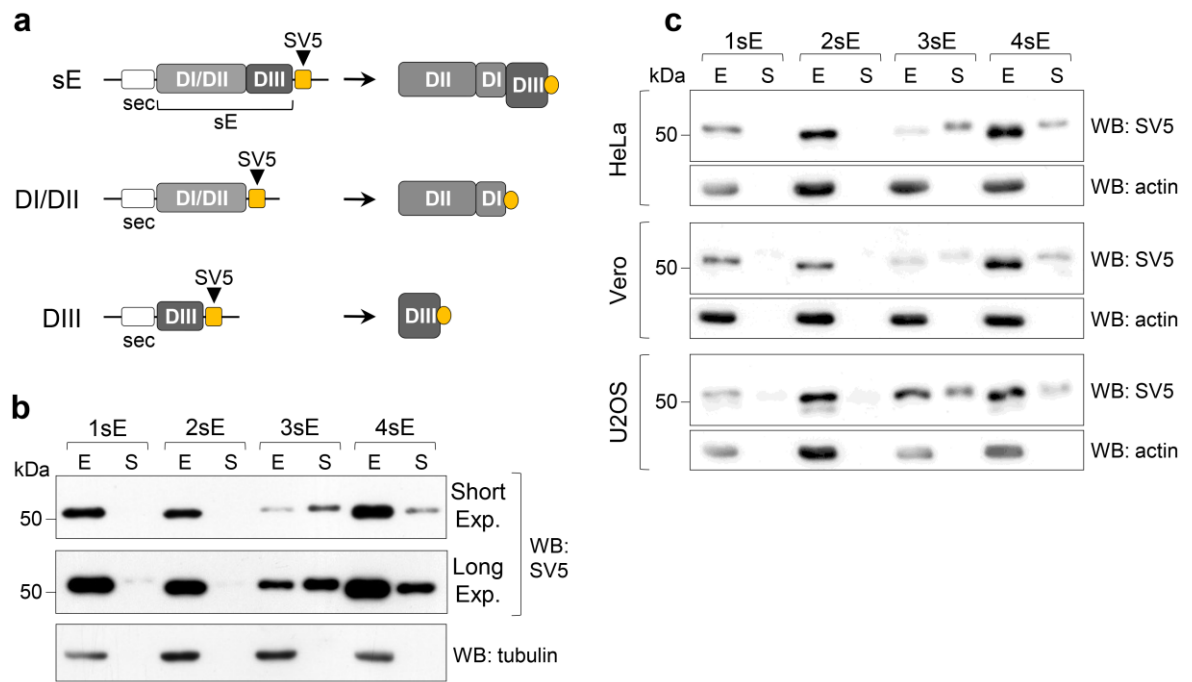


Figure 21. Secretory phenotype of transfected DENV sE proteins from mammalian cells. **a)** Scheme of constructs and their products; sec corresponds to a signal leader peptide and SV5 (orange) to the C-terminal tag. DI/DII and DIII are indicated in light and dark grey respectively. **b)** WB of cellular extracts (E) and supernatants (S) of HEK-293T cells transfected with sE constructs of the indicated serotypes. Short and long exposures are indicated. **c)** WB of cellular extracts (E) and supernatants (S) of HeLa, Vero and U2OS cell lines transfected with sE constructs as in **b**. Anti-tubulin and anti-actin were used as loading controls. (Adapted from reference 439).

To determine if the secretory impairment of 1sE and 2sE was imposed by DI/DII or DIII, we evaluated the secretory properties of constructs that independently expressed these two parts of sE for all four DENV serotypes (Fig. 21a). Fig. 22a shows that plasmids expressing DI/DII of DENV3 and DENV4 (3DI/DII and 4DI/DII, respectively) were efficiently secreted from mammalian cells upon transfection, thus resembling the secretory phenotypes of the corresponding sE. On the other hand, but also reproducing the secretory characteristics of their homologous proteins, DI/DII from serotypes 1 and 2 (1DI/DII and 2DI/DII) were poorly secreted from transfected cells. Although only low amounts of proteins were detected, DIII constructs derived from serotypes 1 and 3 (1DIII and 3DIII), were secreted, while those from serotypes 2 and 4 (2DIII and 4DIII) were not; taken together, these results showed no correlation between the secretory phenotypes of DIII and sE, suggesting that secretion of sE is determined by DI/DII.

This observation was confirmed by designing chimeric sE constructs encoding DI/DII and DIII from different DENV serotypes with opposing secretory behaviours. Four sE chimeras were obtained: i) **1sE(3DIII)** and **2sE(3DIII)**, which encoded the DI/DII regions of poor-secretory DENV serotypes 1 and 2, and the efficiently secreted DIII region of DENV3, respectively; ii) **3sE(2DIII)**, formed by DENV3 secretory DI/DII and the secretory-impaired DIII of DENV2, and iii) **4sE(3DIII)**, combining the secretory DI/DII and the DIII

domains of DENV4 and DENV3 respectively. As demonstrated in Fig. 22b, poor-secretory phenotypes were observed in sE chimeras expressing the DI/DII regions of poor-secretory serotypes DENV1 and DENV2. On the other hand, DI/DII of serotype 3 enabled the secretion of the chimera containing the non-secretory 2DIII. As expected, 4sE(3DIII) was efficiently secreted, and served as a chimeric control for the observed phenotypes. These combinations of profiles allowed us to confirm that the secretory nature of DI/DII defines the behaviour of sE in mammalian cells.

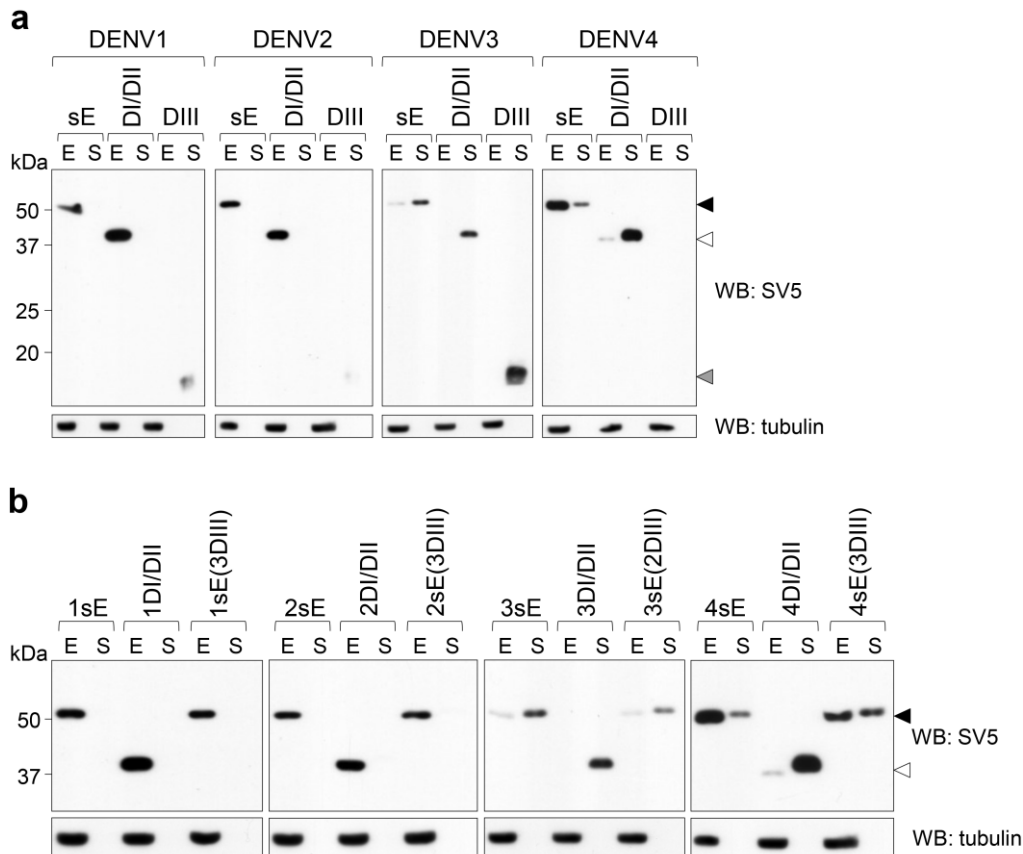


Figure 22. sE secretory phenotype depends on DI/DII. **a)** Cell extracts (E) and culture supernatants (S) of cells transfected with sE, DI/DII and DIII constructs of the indicated serotypes. **b)** WB of cells transfected with the indicated DI/DII-DIII (sE(DIII)) chimeras. For comparison, the corresponding sE and DI/DII proteins were analysed in parallel. Anti-tubulin was used as loading control. Filled, open and grey arrowheads indicate sE, DI/DII and DIII proteins, respectively. (Reprint from reference 439).

To further map the role of DI and DII in determining the secretory phenotype of sE, we obtained chimeric DI/DII constructs by combining DI (including the hinge region) and DII domains from DENV2 and DENV3 which showed opposing secretory phenotypes. As shown in Fig. 23a, the 2DI/3DII chimera containing the DII from secretory DENV3 was well secreted, while the 3DI/2DII, like all constructs expressing 2DII, was not, thus suggesting that sE secretion is DII-dependent. This observation was confirmed by analysing DI and DII chimeras in the context of the whole sE (Fig. 23b). While the chimera expressing 3DI (2sE(3DI)) maintained the poor-secretory phenotype, grafting of 3DII

dramatically improved the secretion of the 2sE-based chimera (2sE(3DII)) (Fig. 23b lanes 3-6). Likewise, transferring 2DI did not affect secretion of the 3sE(2DI) chimera, while expression of 2DII reduced the secretion of the 3sE(2DII) to near-undetectable levels. These results indicate that sE secretory phenotype is strongly determined by DII rather than DI.

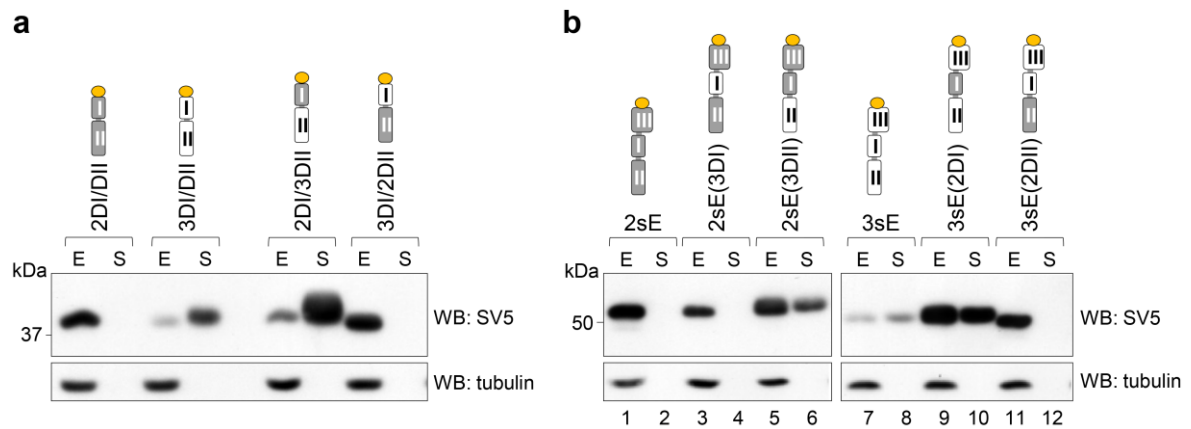


Figure 23. Secretory phenotypes of sE and DI/DII proteins are DII-dependent. WB analysis of cellular extracts (E) and culture supernatants (S) of HEK-293T cells transfected with (a), DI/DII chimeras (2DI/3DII and 3DI/2DII) and (b), sE chimeras (2sE(3DI), 2sE(3DII), 3sE(2DI) and 3sE(2DII)). In both cases, wild type versions of DI/DII and sE proteins are shown as controls; anti-tubulin was used as loading control. (Adapted from reference 439).

Since DII appeared to be fundamental for achieving efficient sE secretion, we used reactivity to monoclonal antibody (mAb) 4G2, which recognises a conformational epitope on the fusion loop of all arthropod-borne flaviviruses⁴⁴⁰, to test the folding state of DII within the different proteins evaluated so far. Positive recognition by 4G2 confirmed the proper folding of DII not only in efficiently secreted DI/DII and sE constructs from DENV3 and DENV4, but also in secreted 2DI/3DII and 2sE(3DII) chimeras (Fig. 24a). Denaturation of the proteins prevented 4G2 recognition, thus confirming the conformational nature of the epitope. In contrast, 4G2 showed no reactivity towards cellular extracts containing DI/DII and sE proteins from poor-secretory DENV1 and DENV2, and not secreted 3DI/2DII and 3sE(2DII) chimeras (3DI/DII and 3sE were included as positive controls) (Fig. 24b). Together, these profiles indicate improper folding of the protein that could explain the poor secretory phenotype of DENV1 and DENV2 in mammalian cells.

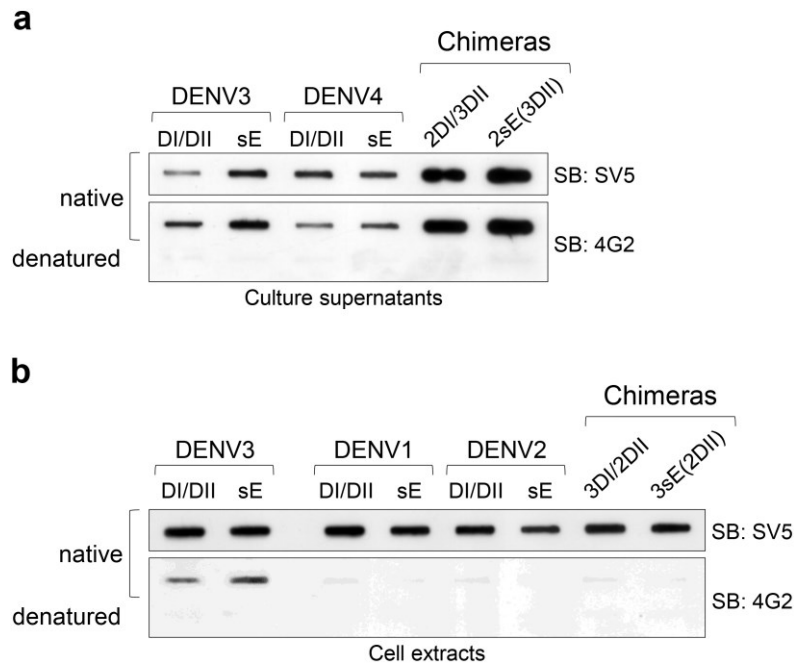


Figure 24. Poor-secretory phenotype of sE and DI/DII proteins is associated with compromised DII folding . Slot blot of (a) culture supernatants of HEK-293T cells transfected with efficiently secreted E-derived constructs, and of (b) cells extracts from cells transfected using poorly-secreted proteins (3sE and 3DI/DII are included as control). Upper panels correspond to native samples reacted with anti-SV5, while lower panels contain to samples in native (upper row) and denatured (lower row) conditions reacted with mAb 4G2. (Reprint from reference 439).

Since E glycosylation was shown to be important for proper trafficking of the viral particles along the secretory pathway^{106,107}, we tested the correlation between 4G2 reactivity and secretory phenotype, using non-glycosylated mutants (Asn67Gln-Asn153Gln) of DENV3-derived DI/DII and sE constructs. As presented in Fig. 25a, contrary to the wild type (wt) proteins both non-glycosylated mutants showed impaired secretion. The wt proteins were treated with PGNase F to demonstrate the removal of glycosylation sites on the mutants. As expected, the non-glycosylated mutants were no longer recognised by 4G2 further confirming the correlation between proper DII folding and efficient secretion from mammalian cells (Fig. 25b).

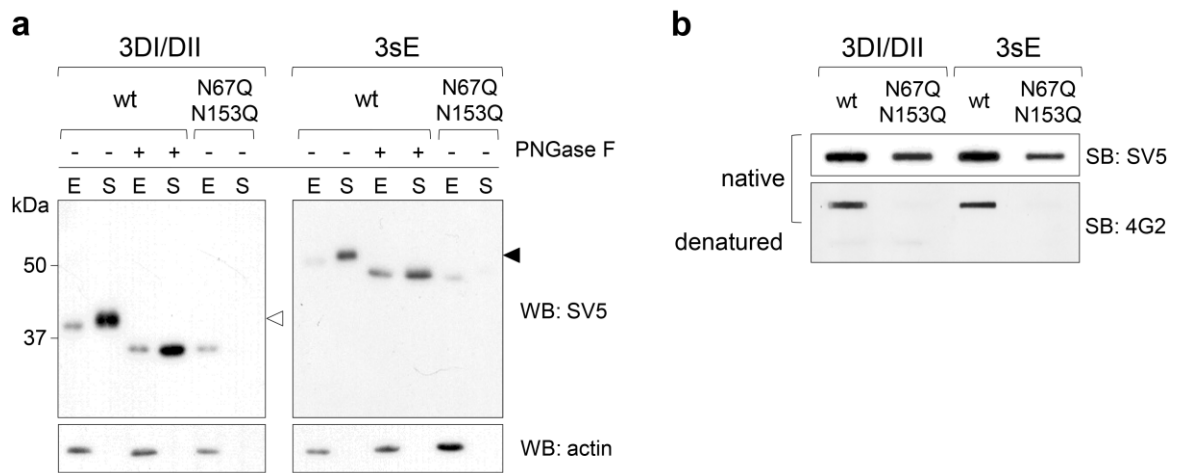


Figure 25. Non-glycosylated DENV3 proteins show impaired secretion and improper DII folding. **a)** WB of cell extracts (E) and culture supernatants (S) of cells transfected with the non-glycosylated 3DI/DII and 3sE mutants (N67Q; N153Q) and the wild type (wt) controls. Samples treated with PNGase F are indicated. Filled and open arrowheads indicate sE and DI/DII proteins, respectively. **b)** Slot blot of cellular extracts containing wt 3DI/DII and 3sE and the non-glycosylated mutants, probed with anti-SV5 or mAb 4G2, on native or denatured samples, as indicated. (Adapted from reference 439).

Interestingly, the secretory phenotype of 2DI/DII showed a temperature-dependent behaviour. Contrary to cell transfection following the “normal protocol” where cells were maintained at 37°C (henceforth referred as 37°C), we tested a “low-temperature protocol” in which cells were maintained at 37°C for 16h after transfection and shifted to 28°C for another 24h following change of culture media (henceforth referred as 28°C) (Fig. 26a). As shown in Fig. 26b, 2DI/DII was efficiently secreted at 28°C but not at 37°C, while the secretory profile of 3DI/DII was not affected. The secretory 2DI/DII formed at 28°C, but not the poor-secretory version produced at 37°C, was recognised by 4G2, consistent with the association between DII folding and secretion (Fig. 26b). Nevertheless, the secretory phenotype of 2sE was not affected by temperature despite the fact that, like 2DI/DII, 2sE produced at 28°C showed positive 4G2 reactivity (Fig. 26a and 26b).

This indicates that, although proper folding is required for efficient secretion, it is not sufficient and other factors restrict secretion under these conditions.

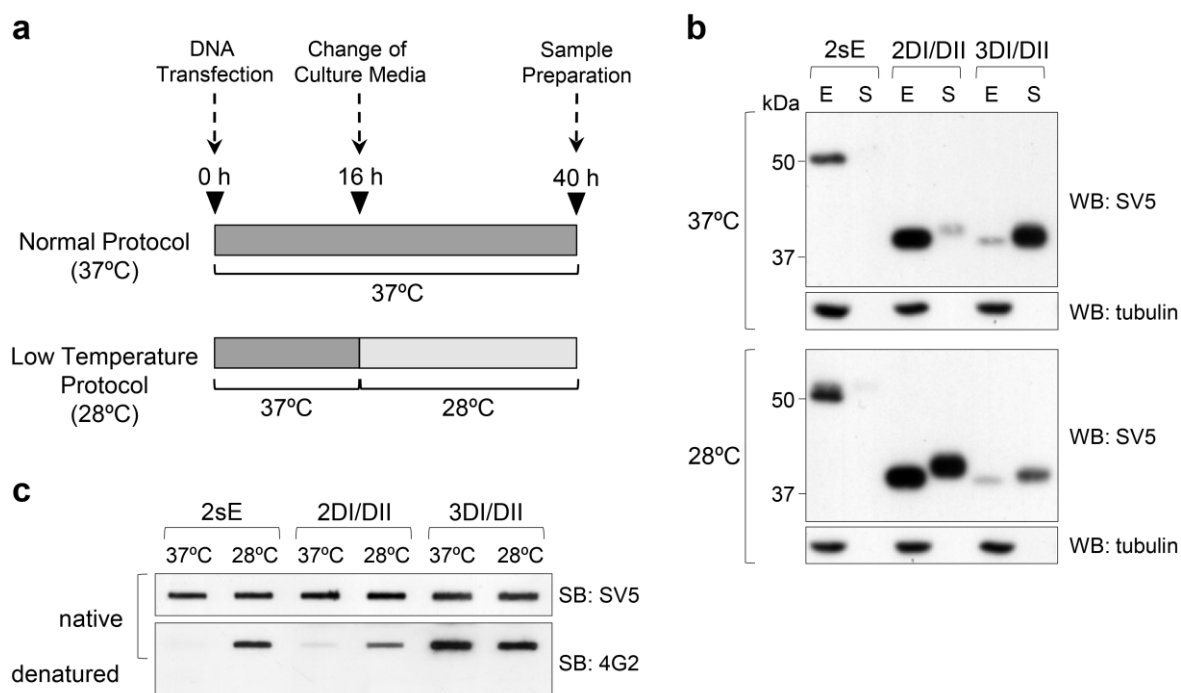


Figure 26. 2DII/DII secretion and folding are temperature-dependent. **a)** Scheme of experimental conditions after transfection indicating temperature shifts for both protocols. **b)** WB of cellular extracts (E) and supernatants (S) of HEK-293T cells transfected with DENV2 sE and DI/DII constructs incubated at 37°C (upper panel) or at 28°C (bottom panel), 3DI/DII is included as control. Anti-tubulin was used as loading control. **c)** Slot blot of cell extracts samples shown in **b**, reacted with anti-SV5 or mAb 4G2, as indicated. (Adapted from reference 439).

1.2.2.sE dimerisation.

1.2.2.1. DIII stabilises sE dimeric interactions.

We then evaluated the ability of secreted sE and DI/DII proteins to dimerise using a cell-based assay to detect dimers by flow cytometry. Display of 3sE on the cell membrane was achieved by designing DNA constructs where the transmembrane and the cytoplasmic domains of the human MHC-I α chain were fused to the C-terminus of 3sE ((m)3sE) (Fig. 27a). Dimerisation was evaluated by co-expressing the (m)3sE construct with SV5-tagged secretory versions of 3sE and 3DI/DII, As revealed using anti-3sE serum, only (m)3sE proteins were displayed on the cell surface after transfection (Fig. 27b). Thus, cells will become positive to anti-SV5 only if the secretory proteins are able to form hetero-dimers with the membrane-bound (m)3sE. Indeed, anti-SV5 reactivity was only observed when cells were co-transfected with (m)3sE and 3sE-SV5 constructs, but not when each construct was used alone (Fig. 27c). In addition, hetero-dimers were also detected when using the 3DI/DII-SV5 construct (Fig. 27d), indicating that stable hetero-dimers between DI/DII and sE were viable. In parallel to cytofluorimetry, transfected cells were also evaluated by immunofluorescence which confirmed the results and revealed the SV5-positive hetero-dimers restricted to the cell surface (Fig. 27e).

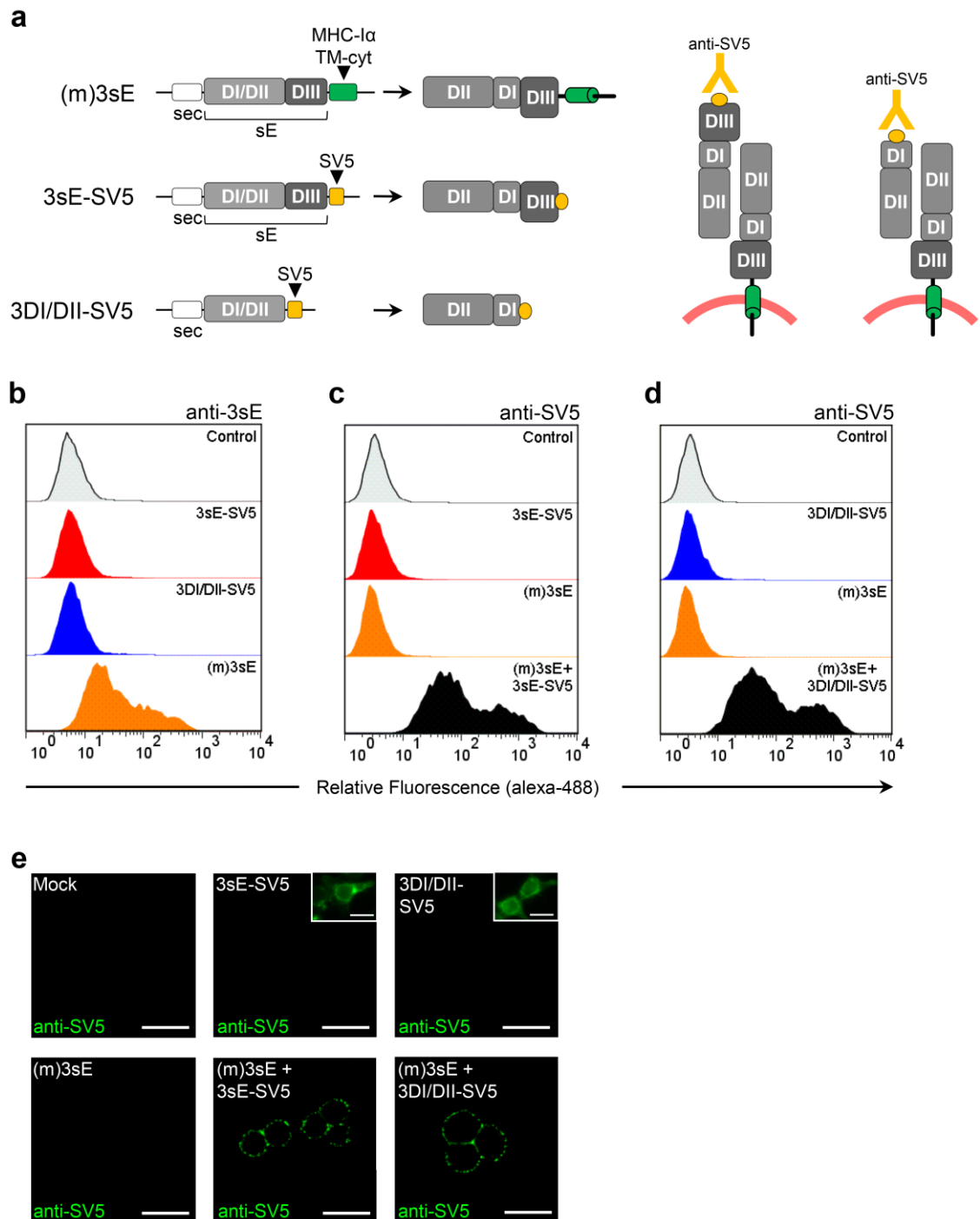


Figure 27. Dimerisation of sE and DI/DII proteins. **a)** Schematic representation of DNA constructs (left) and of the sE-sE homo-dimers and sE-DI/DII hetero-dimers detected on the cell membrane (right). **(b-d)** Cytofluorimetry plots of cells transfected with the indicated constructs and reacted with an anti-3sE serum **(b)**, or with anti-SV5 to detect sE-sE homo-dimers **(c)** and sE-DI/DII hetero-dimers **(d)**. **e)** Immunofluorescence of cells transfected with the indicated constructs and reacted with anti-SV5 to detect homo- and hetero-dimers. Permeabilised controls are shown as inserts within the respective images to highlight intracellular expression of the 3sE-SV5 and 3DI/DII-SV5 constructs. Bar represents 20 μm . (Adapted from reference 439).

Co-immunoprecipitation was used to further confirm the dimerisation of secreted sE constructs. For this, cells were co-transfected with two differently-tagged sE constructs: SV5 or BAP (Biotin Acceptor Peptide, a tag that can be biotinylated *in vivo*, by a biotin-

ligase BirA enzyme active in the ER lumen^{426,427}). After transfection, cell extracts and culture supernatants were immunoprecipitated with anti-SV5 and analysed with HRP-conjugated streptavidin (StrAv) by WB. 3sE, 4sE and the 2sE(3DII) secretory chimera were analysed. Biotinylated sE partners were able to co-immunoprecipitate in all three cases both from cellular extracts (Fig. 28a) and culture supernatants (Fig. 28b). Surprisingly, when analysing 3DI/DII we were able to co-immunoprecipitate the BAP-tagged construct from the cellular extracts (Fig. 28c) but not from the secreted proteins (Fig. 28d), suggesting that DI/DII interactions have low stability and constructs disassociate after secretion.

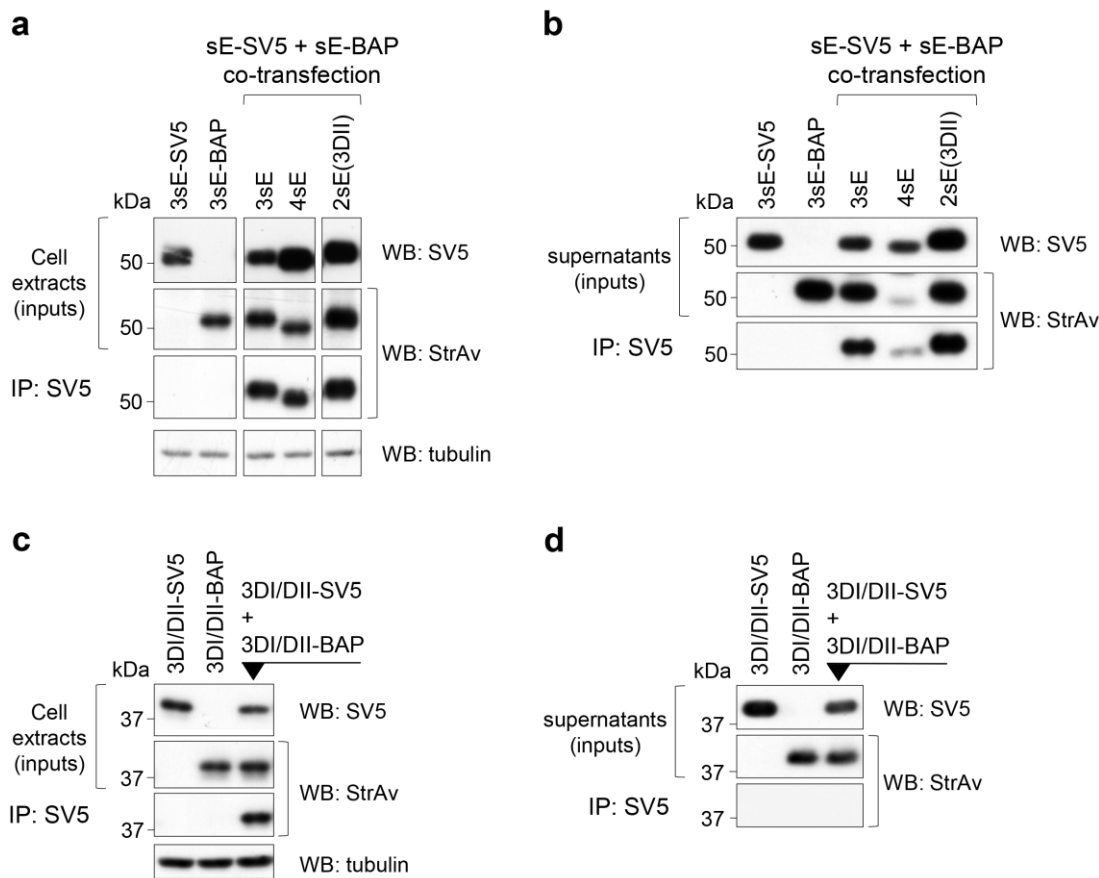


Figure 28. Dimerisation of secreted sE and DI/DII proteins. Co-immunoprecipitations from cellular extracts (**a, c**) or culture supernatants (**b, d**) of HEK-293T cells transfected with the indicated constructs. Immunoprecipitations were carried out with anti-SV5 and co-immunoprecipitated biotinylated partners were detected with HRP-conjugated streptavidin (StrAv). In (**a**) and (**c**) anti-tubulin was used as loading control. (Adapted from reference 439).

Since dimers were only recovered from secreted sE proteins, but not DI/DII, the results indicated that DIII fulfils an important role in stabilising the dimeric interactions among sE monomers. To confirm this, we expanded the immunoprecipitation studies and analysed the interactions between DI/DII and sE constructs in detail. As shown in Fig. 29a, stable dimers (in the form of co-immunoprecipitated BAP-tagged partners) were only observed for 3sE homo-dimers (involving two DIII) and 3sE-3DI/DII hetero-dimers

(involving one DIII), but not for 3DI/DII homo-dimers; the results were independent of whether the pull-down was performed on the sE or DI/DII constructs (Fig. 29a, lanes 6 and 7). The amount of co-immunoprecipitated proteins from the sE-DI/DII hetero-dimers was around four times lower (approximately 5% of input) than the amount of protein recovered from sE homo-dimers (approximately 20% of input) (Fig. 29b), suggesting that the single DIII present in sE-DI/DII interactions is able to stabilise dimerisation to a lower extent than the two DIII present on sE homo-dimers, highlighting the stabilising function of DIII within the sE dimers. To prove the specificity of these interactions we performed an SV5-immunoprecipitation on extracts from [³⁵S]methionine-labelled cells co-transfected with SV5-tagged 3sE and roTag-tagged 3DI/DII. As shown in Fig. 29c, immunoprecipitations of 3sE (with anti-SV5) and 3DI/DII (with anti-roTag) were free of other radioactively-labelled cellular proteins, and anti-SV5 co-immunoprecipitation of 3DI/DII was only observed when co-expressed with 3sE.

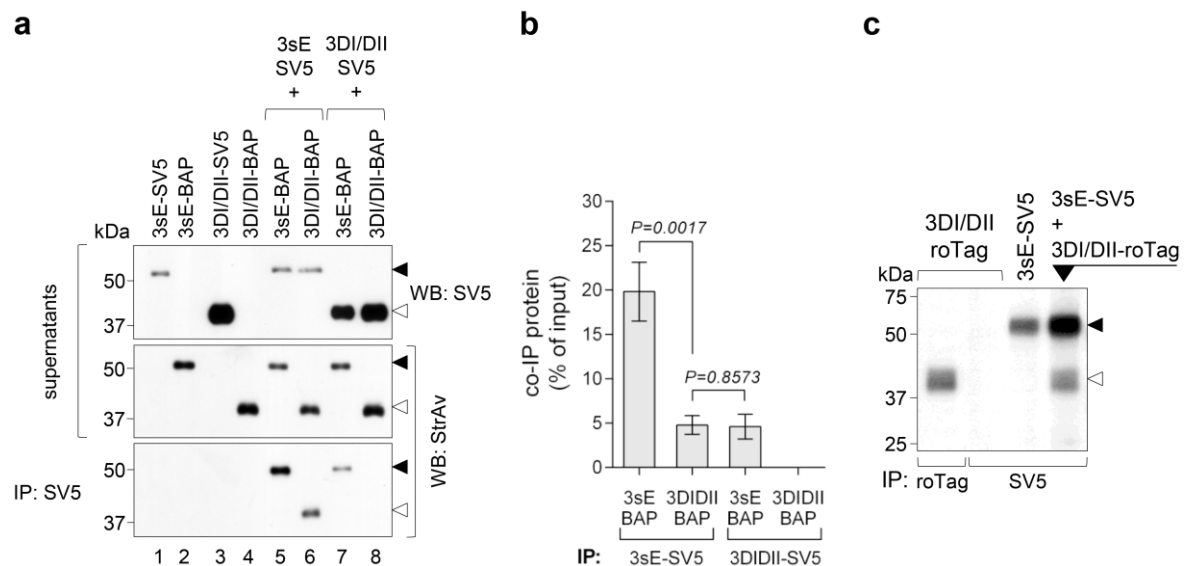


Figure 29. Dimers are stabilised by DIII. **a)** Co-immunoprecipitations of proteins secreted from HEK-293T cells transfected with DENV3 constructs to analyse the formation of homo- and hetero-dimers. The amount of the anti-SV5 immunoprecipitated sample loaded into the gel (bottom panel) corresponds to 10 times the amount of the input (middle panel). **b)** Quantification of the data shown in **(a)** (lanes 5-8), for each combination of co-transfected constructs data is represented as mean \pm s.d. of biological replicates ($n=3$). P values from statistical comparisons between sE-sE homo-dimers and sE-DI/DII hetero-dimers ($t=7,496$, $df=4$), and between variation of sE-DI/DII hetero-dimers ($t=0,1917$, $df=4$), are shown. *, undetected. **c)** Immunoprecipitations with anti-SV5 or anti-roTag of cellular extracts from HEK-293T cells transfected with the indicated constructs and labelled with [³⁵S]Methionine (15 minutes pulse). Filled and open arrowheads correspond to sE and DI/DII, respectively. (Adapted from reference 439).

1.2.2.2. Secretion of covalently stabilised dimeric sE is temperature-dependent.

Taking into consideration the relationship between proper folding, dimerisation and secretion described thus far, and also the temperature-dependent behaviour of 2sE folding, we decided to further investigate how PrM and temperature influence the

production and secretion of sE from the four DENV serotypes and ZIKV in mammalian cells. For this, we designed plasmids encoding the sequence of sE alone, or in the presence of viral proteins M (MsE) and PrM (PrMsE). The SV5 tag was fused to the C-terminal end of sE and to the N-terminus of M or PrM as schematically shown in Fig. 30a. In addition, given the importance of dimer-dependent epitopes in inducing cross-neutralising responses against DENV and ZIKV²⁸⁸, we attempted to stabilise sE dimers by introducing an Ala to Cys mutation in the inner surface of DII (Ala259 in sE from DENV1, 2 and 4; Ala257 in DENV3 and Ala264 in ZIKV) (Fig. 30b). If sE truly undergoes transient dimerisation in the secretory pathway as our previous results indicate, the introduced cysteine would face itself in the opposite monomer, forming a disulphide bond stabilising the dimeric sE.

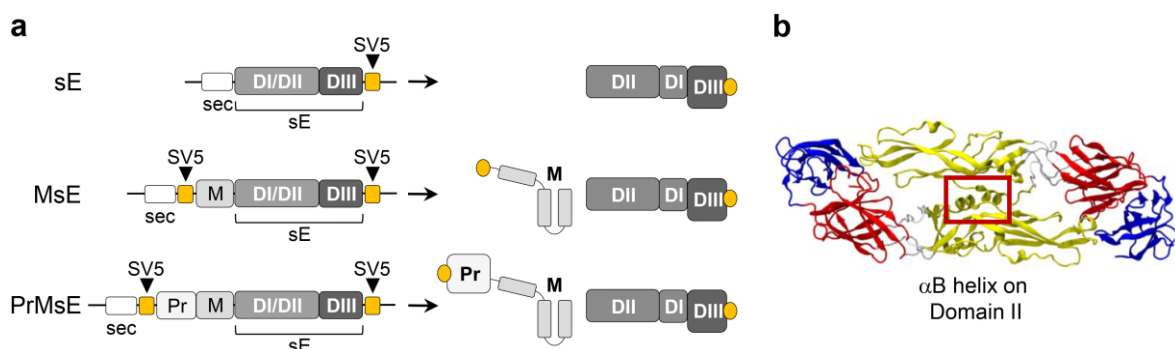


Figure 30. a) Schematic representation of DNA constructs and expected products. **b)** E dimeric structure of DENV3 (PDB reference 1uzg) indicating the position where the Ala257Cys mutation was introduced.

HEK-293T cells were transfected using constructs with or without M and PrM, expressing the wild type (sE-wt) or mutant sE from DENV2, DENV3 and ZIKV (ZsE), following the temperature protocols described in Fig. 26a. Expression and secretion of the encoded proteins was analysed by non-reducing WB of cellular extracts and culture supernatants from transfected cells. Results from experiments incubated at 37°C and 28°C are shown in Fig. 31a and Fig. 31b respectively.

At 37°C, 3sE was secreted from both wild type and mutated constructs, however, while the secreted sE protein from 3sE-wt constructs run as a monomer, the Ala257Cys mutant was found as a covalent dimer (sE-cvD) (Fig. 31a, leftmost panel). Moreover, 3sE-wt and 3sE-cvD were equally produced and secreted regardless of the presence of PrM (or M). In contrast, for DENV2 and ZIKV (Fig. 31a, centre and rightmost panels, respectively), sE-wt and sE-cvD proteins were not secreted with any of the constructs tested; instead a smear of disulphide bonded folding intermediates was detected intracellularly. At 28°C (Fig. 31b), however, sE-wt and sE-cvD of DENV3 and ZIKV were efficiently produced and secreted from transfected cells, while 2sE, in agreement with our previous results, was only secreted from the 2sE-cvD mutant but not from the 2sE-wt constructs. In all cases, the results were independent of the presence of PrM and highlight

the fundamental role of temperature for inducing proper folding and dimerisation of sE proteins in mammalian cells.

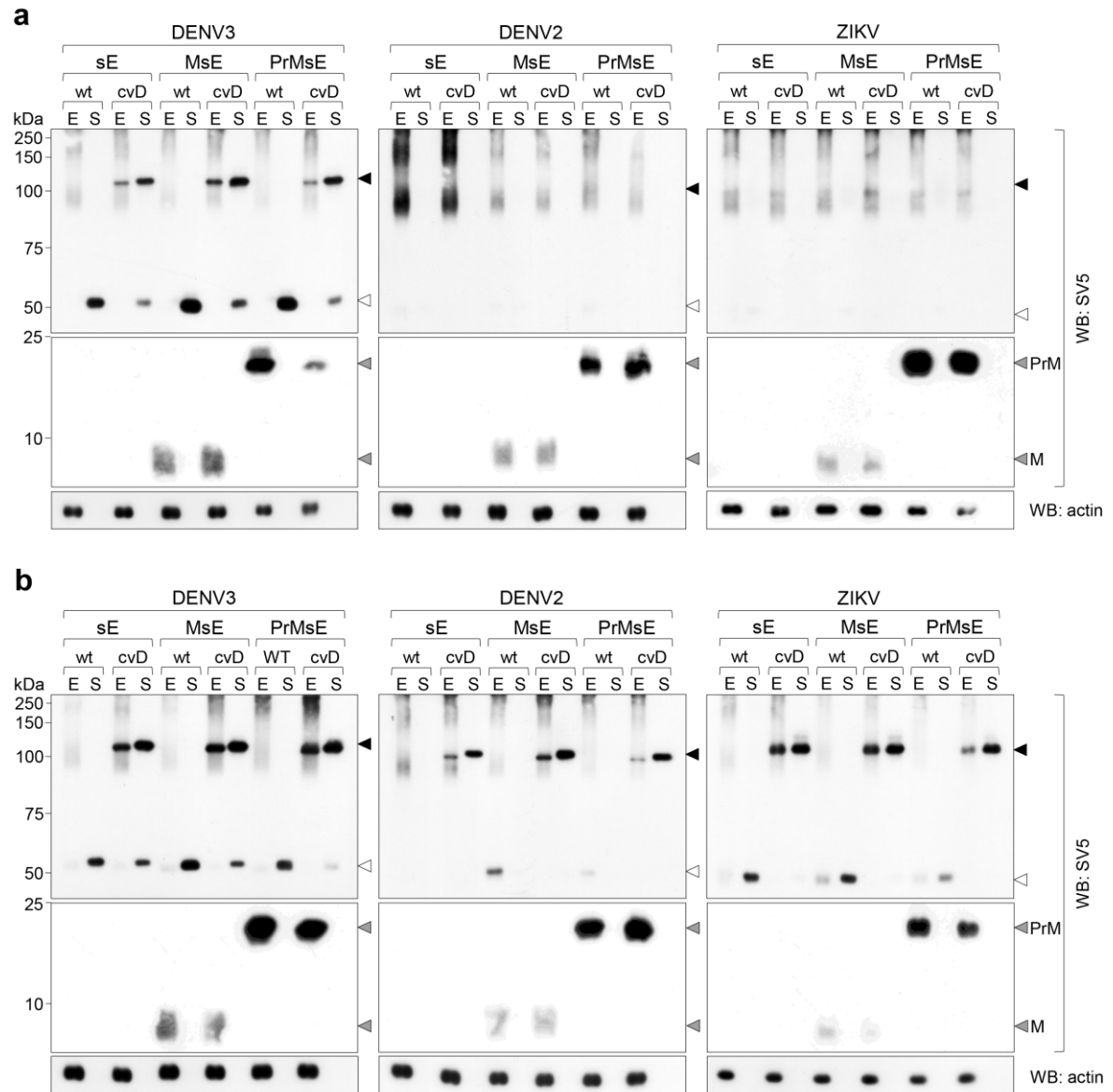


Figure 31. Secretory profiles of covalently stabilised sE dimers of DENV and ZIKV in mammalian cells. Non-reducing WB of total cell extracts (E) and culture supernatants (S) of HEK-293T cells transfected with the indicated constructs and incubated at 37°C (a) or at 28°C (b). Anti-actin was used as loading control. Open and filled arrowheads indicate monomeric and dimeric sE, respectively.

For DENV1 and DENV4, temperature-dependent dimerisation and secretion of sE-cvD constructs, in absence of PrM, was also confirmed (Fig. 32a). Moreover, a temperature-dependent secretory phenotype was also observed for sE-wt of DENV1 that, just as initially described for the 2DI/DII constructs, was efficiently secreted only from transfected cells at 28°C. Using constructs coding for the sE variants of DENV3 and DENV2 we also determined that the temperature-dependent and PrM-independent behaviour was also independent of the mammalian cell line used for transfection (Fig. 32b).

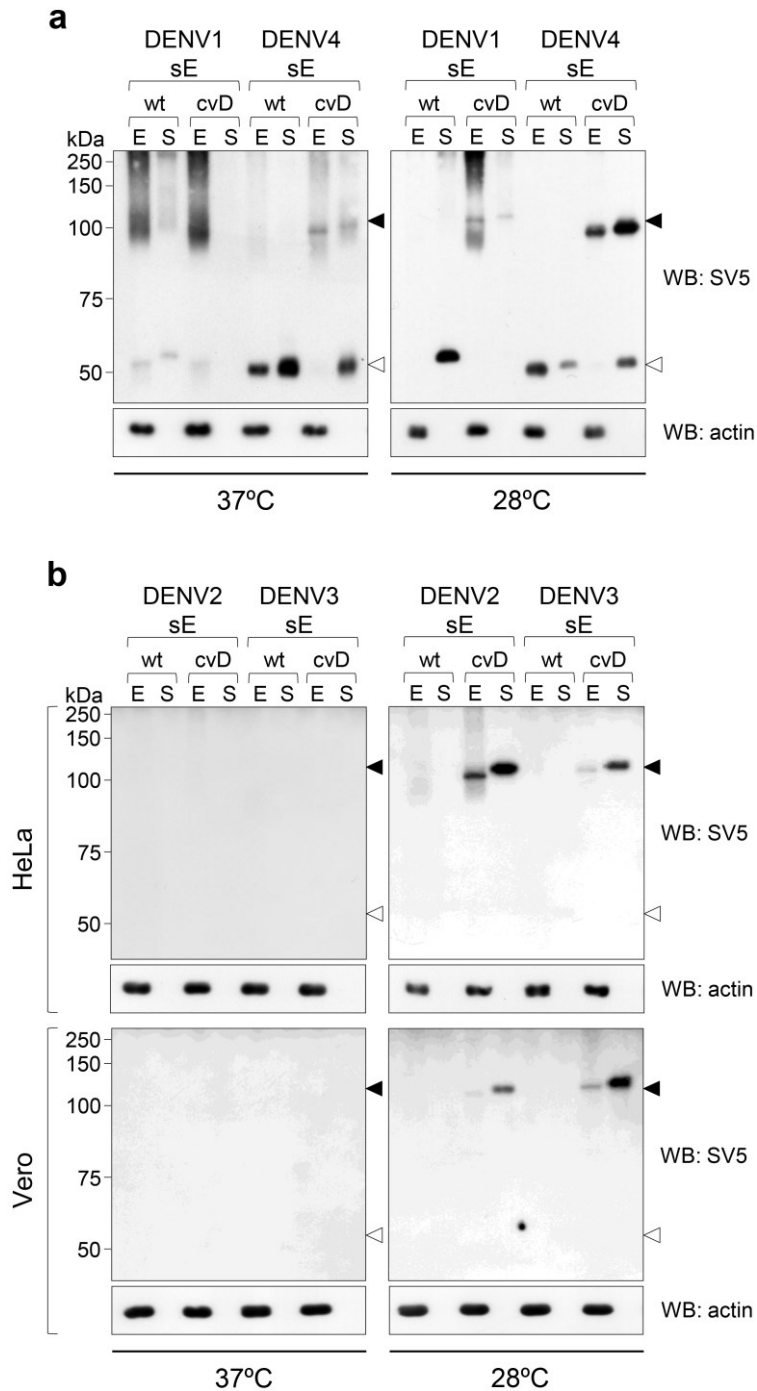


Figure 32. Secretory profiles of covalently stabilised DENV sE dimers of different mammalian cells. a) Non-reducing WB of cell extracts (E) and culture supernatants (S) of HEK-293T cells transfected with sE-wt and sE-cvD constructs of DENV1 and DENV4, incubated at 37°C (left panel) or at 28°C (right panel). Open and filled arrowheads indicate monomeric and dimeric sE, respectively. **b)** Dimerisation and secretion of sE-wt and sE-cvD of DENV2 and DENV3 in HeLa (top) or Vero cells (bottom), incubated at 37°C or 28°C, as indicated. Anti-actin was used as loading control.

ELISA and cytofluorimetry were used to evaluate the proper folding and assembly of sE-cvD constructs. For ELISA, secreted biotinylated covalent sE dimers from DENV2, 3 and 4, and ZIKV were obtained from the supernatant of transfected HEK-293T cells using the BAP-tag/BirA biotinylation system described before (DENV1 was excluded from the

assay as the amount of secreted 1sE-cvD was not enough to perform a comparable assay), captured in avidin-coated plates and probed with a series of mAbs targeting different conformational epitopes: 4G2 and 4E5A (which targets a DENV-specific epitope on DIII^{441,442}) recognise epitopes restricted to one domain present on a single E protein; EDE1-C10, EDE2-B7 (against dimeric epitopes EDE1 and EDE2, respectively²⁸⁴), 2D22 (recognising also a dimeric epitope specific of DENV2²⁸⁵) and 5J7 (a DENV3-specific antibody whose footprint involves binding to 3 different E proteins from two adjacent E dimers on the viral surface²⁸¹), bind to quaternary epitopes involving more than one E molecule. In addition, mAb 1F4, a DENV1-specific antibody recognising residues on DI and hinge regions of a single E monomer as displayed on the viral surface, but not on 1sE recombinant proteins obtained from insect cell lines²⁸², was also included. The results for the ELISA analysis are shown in Fig. 33, EDE-specific mAbs EDE1-C10 and EDE2-B7 were able to recognise cvD versions of 2sE, 3sE and 4sE, while only EDE1-C10 reacted positively with ZsE-cvD as ZsE was shown to be recognised less efficiently by anti-EDE2 antibodies^{288,289}. As expected, 2D22 recognised only 2sE-cvD, while 4E5A reacted with all DENV-derived proteins containing DIII and 4G2 was only negative against the control 4DIII. Since DENV1 proteins were not included 1F4 did not recognise any of the tested proteins; likewise, the lack of higher order DENV3 E dimers arrangements explains the non-reactivity of mAb 5J7.

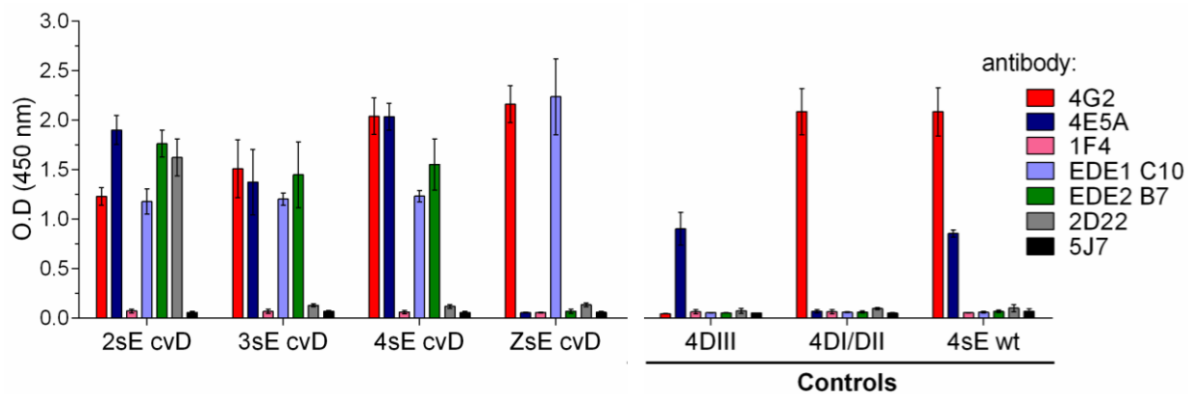


Figure 33. Structural analysis of covalently stabilized E dimers from DENV serotypes 2, 3 and 4 and ZIKV in ELISA with the indicated mAbs (left panel). Control antigens, 4DIII, 4DI/DII and 4sE wt were also included (right panel); data is represented as mean±s.d. (in all cases, $n=5$).

The same selection of mAbs (with an additional EDE1-specific antibody, EDE1-C8²⁸⁴) was then used to analyse membrane displayed versions of wt ((m)sE-wt)^c and cvD ((m)sE-cvD) constructs, following transfection of HEK-293T cells at 28°C and 37°C. The reactivity profiles for (m)sE-cvD (Fig. 34) and (m)sE-wt (Fig. 35) proteins, resembled those described for the ELISA with three important differences: i) the (m)sE-wt proteins of the

^c There are no differences between the (m)sE constructs described in the previous section and the (m)sE-wt constructs mentioned here. The “-wt” termination is added to highlight the differences with the proteins carrying the cvD mutation.

four DENV serotypes and ZIKV were displayed as dimers, as evidenced by the positive reactivity to dimer-specific mAbs, ii) mAb 1F4 was positive with both 1sE formats (-wt and -cvD) and iii) mAb 5J7 reacted with DENV3 sE-cvD, but not with sE-wt. We therefore concluded that the secreted and membrane bound versions of sE-cvD constructs replicate the structure of the antiparallel E dimers present on the viral surface.

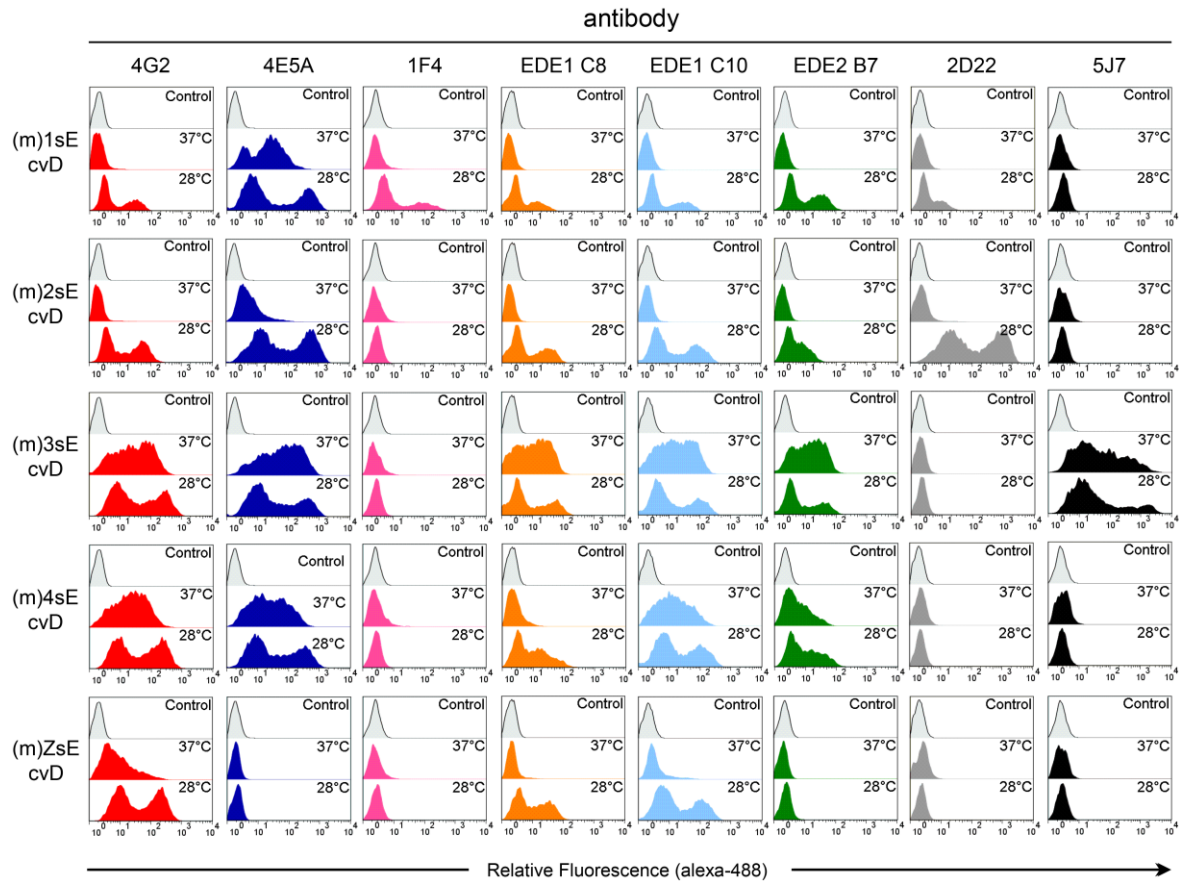


Figure 34. Cytofluorimetric analysis of HEK-293T cells transfected with (m)sE-cvD constructs of all four DENV serotypes and ZIKV at 37°C or 28°C, and reacted with the indicated mAbs. Mock-transfected HEK-293T cells incubated with each mAb served as controls.

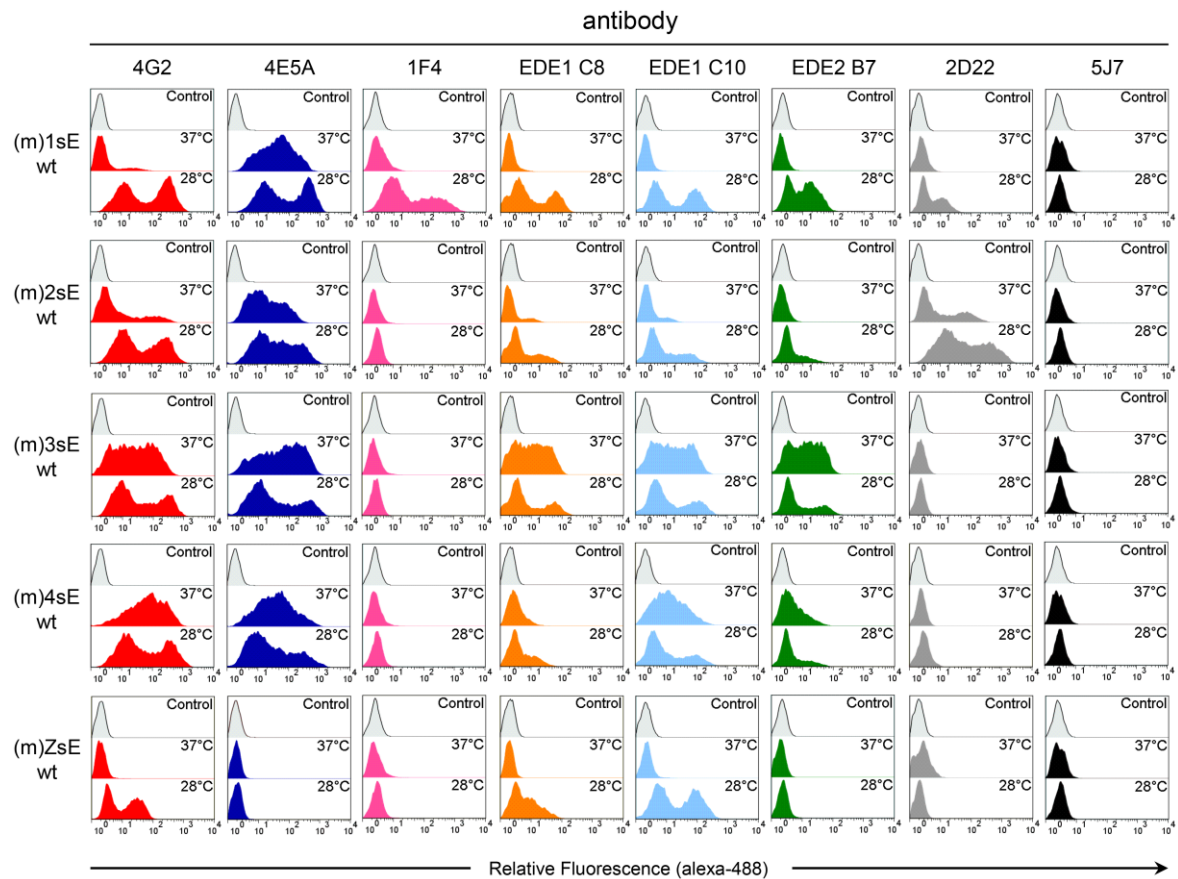


Figure 35. Cytofluorimetric analysis of HEK-293T cells transfected with (m)sE-wt constructs of all four DENV serotypes and ZIKV at 37°C or 28°C, and reacted with the indicated mAbs. Mock-transfected HEK-293T cells incubated with each mAb served as controls.

1.2.2.3. Covalent stabilisation of E dimers on the viral surface.

We then attempted expressing the covalent dimeric E mutants on the surface of viral particles. We used a pseudoviral approach based on a previously described WNV replicon encoding a reporter EGFP (WNV-rep⁴³⁶). Using this system, infectivity of pseudoviral particles can be assessed by determining the number of EGFP-positive cells following infection. Since ZIKV structural proteins have been shown to efficiently package WNV-rep⁴³⁷, the objective was to produce pseudoviruses using constructs containing ZIKV-derived C-PrME structural proteins carrying the E-wt sequence (ZE-wt) or the Ala264Cys E-cvD mutation (ZE-cvD). If covalent dimers were successfully assembled and incorporated into pseudoviruses, infectivity of the produced particles should be compromised since E trimerisation, and thus the fusion of the viral and endosomal membranes, would be blocked by the disulphide bond. On the other hand, pseudoviruses with the wt sequence of ZIKV E should be infective, allowing detection of EGFP from infected cells, as shown in Fig. 36.

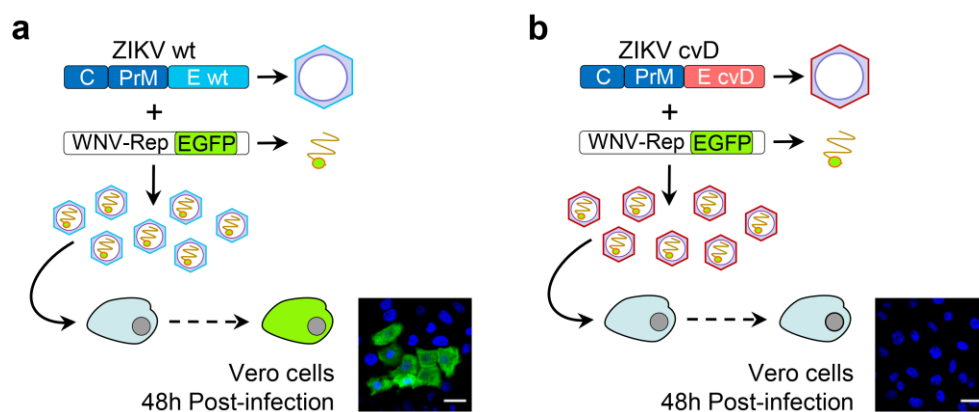


Figure 36. Production of pseudoviral particles using E-wt and E-cvD proteins. Diagrams of the ZIKV-wt (a) and -cvD (b) constructs and the WNV replicon used to produce pseudoviral particles, tested for infectivity (EGFP expression) on Vero cells. Images correspond to representative experiments (Bar, 30µm).

Production of pseudoviral particles was determined by RT/PCR of the WNV-rep RNA from culture supernatants of transfected cells. As shown in Fig. 37a, WNV-rep RNA was successfully amplified from culture supernatants of cells co-transfected with either ZE-wt or ZE-cvD and the WNV-rep, but not from cells transfected with the WNV-rep alone. Cells transfected only with packaging plasmids were used as controls. Consistent with the temperature-dependent behaviour of ZsE-cvD proteins, production of pseudoviruses with ZE-cvD was more efficient at 28°C, while the yield of particles with ZE-wt showed no significant differences between 28°C and 37°C. Moreover, non-reducing WB analysis of pseudoviral preparations confirmed detection of the dimeric form of the full E-cvD protein at 28°C, while the E-wt protein, as expected, run as a monomer (Fig. 37b). As expected, all the protein recovered from the cvD pseudoviruses was dimeric.

As expected, pseudoviral particles prepared with ZE-cvD were not able to infect Vero, HuH-7 or HEK-293T cells, while wt particles did (Fig. 37c). To exclude that the lack of infectivity of ZE-cvD pseudoviruses was due to compromised interaction with cellular receptors, we performed an ADE assay on K562 cells as antibody-mediated infection should bypass entry involving viral receptors on the cell surface. As shown in Fig. 37d, infectivity of wt pseudoviruses was enhanced by anti-DI/DII antibodies, while cvD pseudoviruses remained non-infective, consistent with impaired membrane fusion within the endosome due to lack of E trimerisation of cvD mutants. Collectively, these results indicate that viral particles with covalently-bound E protein dimers are permissive for assembly in the ER and compatible with trafficking through the secretory pathway.

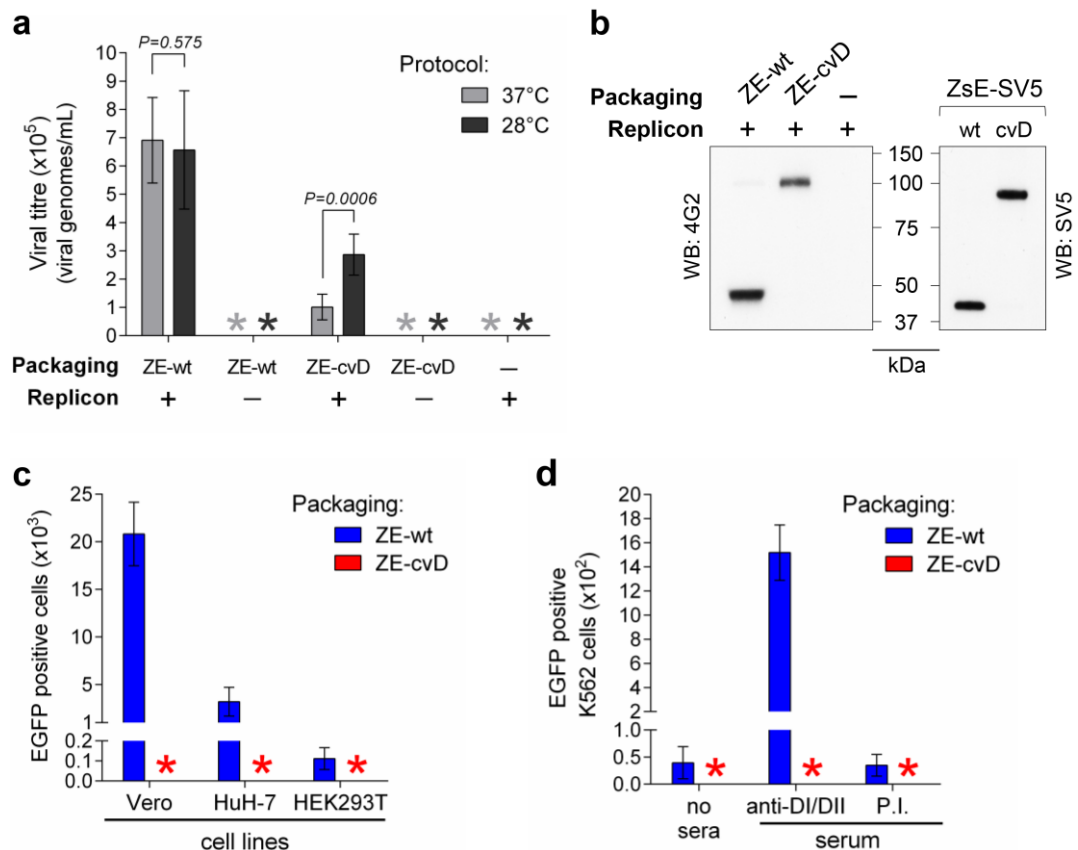


Figure 37. Assemble of ZE-cvD proteins into pseudoviral particles. **a)** Relative quantification of pseudoviruses determined by RT/PCR of the replicon-derived RNA, on samples obtained with ZE-wt packaging construct with ($n=6$; $t=0.5790$ $df=10$) and without ($n=6$) replicon, or ZE-cvD packaging construct with ($n=6$; $t=4.896$ $df=10$) and without replicon ($n=6$), supernatant of cells transfected only with the WNV replicon were used as control, data is represented as mean \pm s.d. **b)** Left panel, non-reducing WB of pseudoviral preparations obtained with the ZE-wt or ZE-cvD packaging constructs developed with mAb 4G2, culture supernatant of cells transfected only with the WNV replicon is shown as control. Right panel, WB of the corresponding SV5-tagged proteins developed with anti-SV5; migration differences correspond to the effect of the stem-anchor region in pseudoviruses. **c)** Infectivity of pseudoviral particles produced at 28°C with ZE-wt or ZE-cvD packaging constructs on Vero ($n=5$), HuH-7 ($n=5$) and HEK-293T cells ($n=5$), determined as EGFP positive cells at 48 h post-infection using 2×10^4 viral particles (as quantified by RT/PCR). **d)** Infectivity of same pseudoviral particles as in **(c)** on K562 cells in the absence of serum ($n=5$) or in the presence of an anti-DI/DII serum ($n=5$) or a negative control pre-immune serum ($n=5$). In all cases, data is represented as mean \pm s.d. *, undetected.

1.2.2.4. Dimerisation of heterotypic sE proteins.

Since assembly and secretion of sE-cvD proteins were viable for all DENV serotypes and ZIKV, we tested whether sE proteins from different viruses were able to interact with each other and form covalent hetero-dimers. For this, we designed sE-cvD constructs carrying two different tags to distinguish between the interacting monomers following a similar approach used for the experiments involving immunoprecipitations (Fig. 28). 3sE was tagged with BAP, while 1sE, 2sE, 3sE (as control), 4sE and ZsE were tagged with SV5. HEK-293T cells were then co-transfected with BAP-tagged 3sE-cvD and each one of

the SV5-tagged cvD proteins. As outlined in Fig. 38a, the strategy for discrimination between homo- and hetero-dimers was based on the detection of the different tags and migration patterns of the constructs on non-reducing WB. The results indicate that 3sE efficiently formed secreted E covalent dimers with 2sE, 4sE and ZsE (Fig. 38b), while 3sE-1sE hetero-dimers were also formed and secreted although in lower amounts (Fig. 38b, left panel).

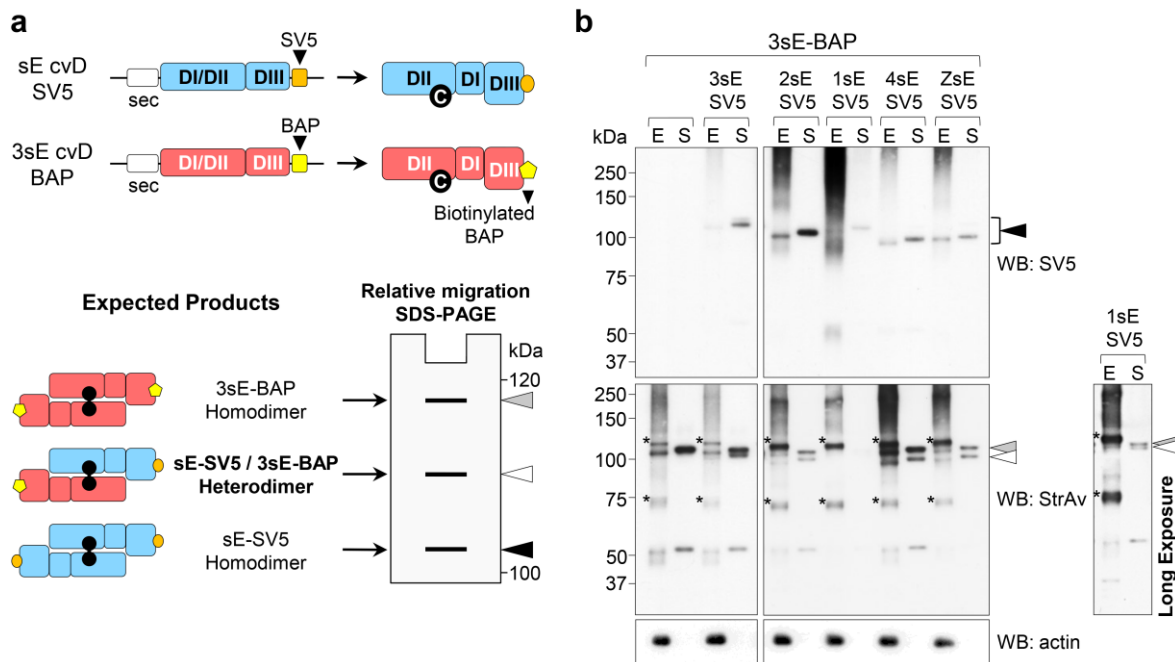


Figure 38. Hetero-dimerisation of E proteins from different flaviviruses. **a)** Approach used to detect E hetero-dimers by WB. For all DENV serotypes and ZIKV, SV5-tagged sE constructs were used, while 3sE was also tagged with BAP and co-expressed with the SV5-tagged proteins. Relative migration in non-reducing PAGE of expected homo- and hetero-dimers and their schematic representation are indicated. **b)** Non-reducing WB of cell extracts (E) and culture supernatants (S) of HEK-293T cells co-transfected with the indicated constructs and developed with anti-SV5 (upper panel) or StrAv-HRP (lower panel). * indicates biotinylated host intracellular proteins. Arrowheads indicate migration of dimers as shown in (a). Anti-actin was used as loading control. Open arrowheads indicate hetero-dimers. Right panel, long exposure of poorly secreted 1sE-3sE hetero-dimers.

The WB results were confirmed by cytofluorimetry using the same cell-based assay used to evaluate the role of DIII in 3sE dimerisation (Fig. 27a). (m)sE-cvD constructs for the four DENV serotypes and ZIKV were co-expressed with secretory, SV5-tagged 3sE-cvD in mammalian cells. As before, transfected cells stained positively for the SV5 tag only when the secretory protein was retained in the cell membrane following hetero-dimerisation with the membrane displayed constructs. As observed in the WB analysis, 3sE-cvD hetero-dimerisation was observed with all the (m)sE-cvD constructs (Fig. 39a). Interestingly, hetero-dimers were also produced when the experiment was performed using the sE-wt proteins, even though the interaction was significantly less efficient with (m)1sE-wt and (m)4sE-wt, and almost unnoticeable with (m)ZsE-wt (Fig. 39b). Together,

these results indicate that interaction between heterotypic E proteins is not imposed by the disulphide bridge in cvD mutants but rather a consequence of spontaneous interactions between monomers.

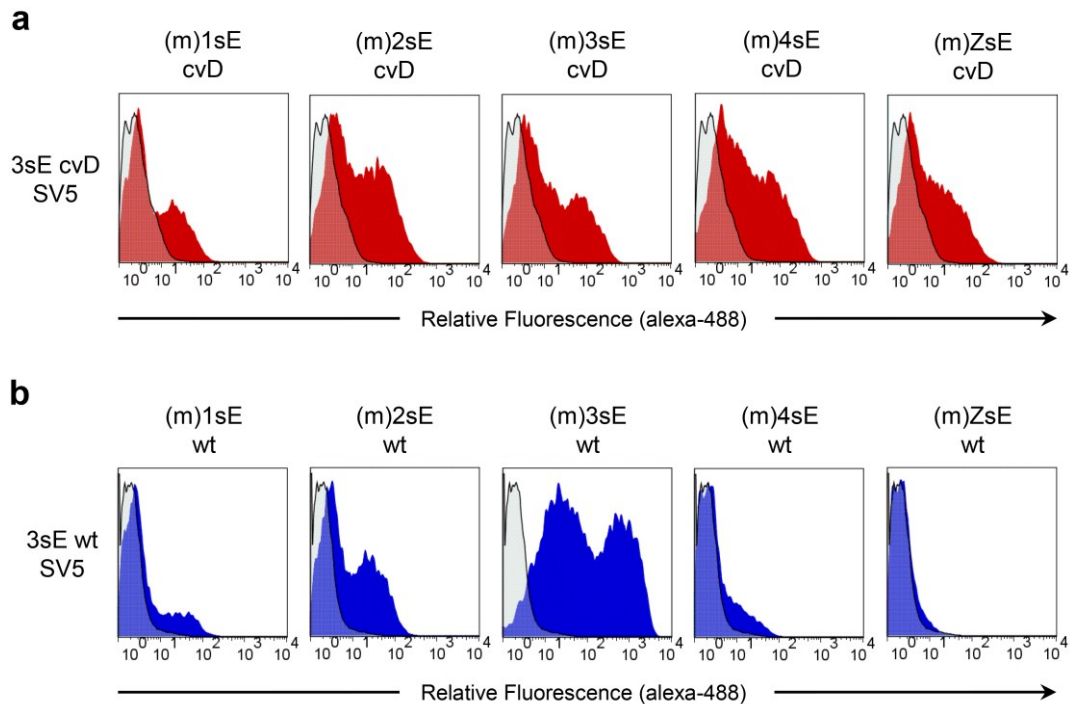


Figure 39. sE Hetero-dimerisation is not imposed by the cvD mutation. Cytofluorimetry profiling of HEK-293T cells co-transfected at 28°C with the membrane displayed (m)sE-cvD proteins and the secretory SV5-tagged 3sE-cvD **(a)** or with the (m)sE-wt proteins and the secretory SV5-tagged 3sE-wt **(b)** of all four DENV serotypes or ZIKV. Profiles correspond to anti-SV5 reactivity and are compared to HEK-293T cells transfected only with the secretory SV5-tagged 3sE.

1.2.2.5. E mosaic viruses.

After demonstrating that hetero-dimerisation was permissive in the context of secreted sE proteins, we decided to assess the possibility of producing viral particles with “mosaic” surfaces formed by distinct E proteins. Using the aforementioned pseudoviral approach, we attempted to package the WNV-rep using constructs expressing ZIKV and DENV E proteins. Following the transfection protocol at 37°C, production of infective pseudoviruses was possible using the ZE-wt construct, but not with a chimera coding for ZIKV C protein and DENV2 PrME (C^Z -PrME^{DENV2}) (Fig. 40a, panels 1 and 2). In the same way, infective particles were not obtained when transfecting ZIKV-C and DENV2-PrME in *trans* (Fig. 40a, panel 3). In contrast, infective pseudoviruses were recovered after co-transfection of ZE-wt and DENV2-PrME (Fig. 39a, panel 4) although, as evidenced by Vero-cell infectivity, the production efficiency was lower when compared to transfection of ZE-wt alone (Fig. 40b).

To determine if pseudoviruses produced using E proteins from ZIKV and DENV2 presented a mosaic E display on their surface, we designed an ADE assay in which

infection was enhanced using a specific combination of antibodies (Fig. 40c). DENV2-specific antibodies, such as those directed against DENV2 DIII (as thoroughly demonstrated in the following sections) should only promote the infectivity of particles displaying DENV2 E protein on their surface, while cross-reacting antibodies, such as those directed against DI/DII, should induce ADE on both ZIKV-only and DENV2-ZIKV mosaic pseudoviruses. Indeed, anti-2DIII antibodies specifically enhanced the infectivity of particles produced by co-expressing both E proteins, while pseudoviruses produced with ZE-wt only were not significantly affected. As expected, significant enhancement on both types of pseudoviruses was observed when using anti-DI/DII antibodies, while pre-immune control sera had no significant effect on infectivity (Fig. 40d). Therefore, DENV2 E protein was (partially) incorporated on the viral surface when co-expressed with the ZIKV structural proteins, producing particles with a mosaic E composition.

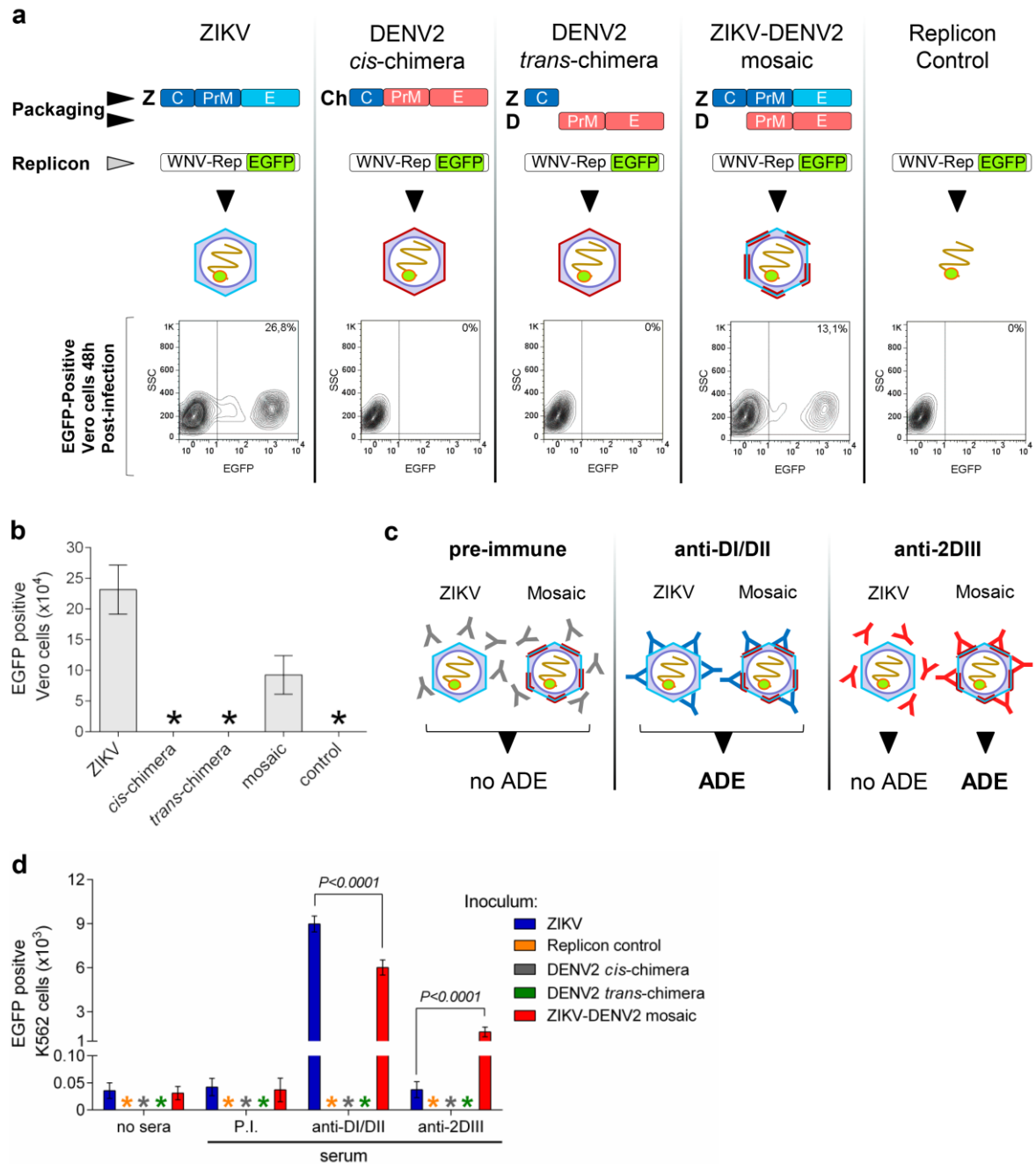


Figure 40. Assembly of mosaic pseudoviruses with E proteins of ZIKV and DENV2. **a)** Production of pseudoviruses by co-transfection of the WNV replicon and different packaging constructs, and revealed by EGFP expression in Vero cells 48h post-infection. Percentages of EGFP positive cells are indicated. **Z**, ZIKV; **Ch**, chimeric; **D**, DENV2. **b)** Total number of infected Vero cells obtained using equal volumes of pseudoviral preparations ($n=5$), data is represented as mean \pm s.d. **c)** Expected ADE activity for particles packaged with only ZIKV protein or ZIKV/DENV2 mosaic particles incubated with antibodies recognising both, ZIKV and DENV2 E proteins (anti-DI/DII) or only DENV2 E protein (anti-2DIII). **d)** EGFP positive K562 cells following 48h infection with supernatants of transfections as in **(a)**, and treated with anti-DI/DII ($n=5$ for each preparation; $t=8,868$ $df=8$), anti-2DIII ($n=5$ for each preparation; $t=10,70$ $df=8$) or control antibodies ($n=5$), Equal volumes of inocula were used to infect cells, data is represented as mean \pm s.d. *, undetected.

1.2.3. Antigen secretion and immune response upon DNA vaccination.

It has been proposed that, for genetic vaccines, induction of an efficient antibody response depends heavily on proper antigen secretion from transfected cells^{395,443}. Therefore, in the case of DENV DNA vaccines, formulations encoding thoroughly studied antigens with a well-established secretory phenotype from transfected cells, would improve antigen availability and induce stronger antibody responses. We were able to confirm this hypothesis by gene-gun DNA immunisation of mice using two groups of plasmids encoding sE proteins that share the same DIII but have different secretory phenotypes in mammalian cells: i) 3DIII was expressed in the context of the efficiently secreted 3sE and the poor-secretory 3sE(2DII) chimera; while ii) 2DIII was included within the poorly-secreted 2sE and the secretory 2sE(3DII) chimera (secretory phenotypes of these constructs are shown in Fig. 23b). Following vaccination, anti-DIII titres were measured by ELISA using recombinant monobiotinylated DIII of DENV2 and DENV3 for coating. Since antibodies directed against DI/DII were not detected due to the DIII-specific coating of the ELISA plates, and the 3DIII and 2DIII regions expressed in each vaccination group were identical, the assay allowed us to determine how antigen secretion from transfected cells influenced the anti-DIII response. Animals immunised with the secretory 3sE protein developed an anti-3DIII titre above 2000, while those that received the poor-secretory 3sE(2DII) chimera were negative at 1/100 dilution (Fig. 41a). As with the 3DIII-expressing constructs, the anti-2DIII titres were ten times higher in the group immunised with the secretory 2sE(3DII) chimera than in the group vaccinated with 2sE (Fig. 41b). Cross-reactivity of 3DIII-based sera against 2DIII, and the 2DIII-based sera against 3DIII was very low (Fig. 41c and 41d, respectively). Antibody titres against the homologous DIII are summarized in Fig. 41e, and further highlight how antigen availability (in the form of secreted antigen) determines the strength of the antibody responses. As shown by the immunofluorescence in Fig. 41f, anti-2sE, anti-2sE(3DII) and anti-3sE sera were able to recognise the mature viral protein in virus-infected cells. In all cases, pre-immune sera were used as negative control^d.

^d Sera from non-immunised mice (Pre-immune sera) were as negative as sera from mock-immunised animals, i.e. mice immunised with the empty pcDNA3.1 or pVAX1 vector.

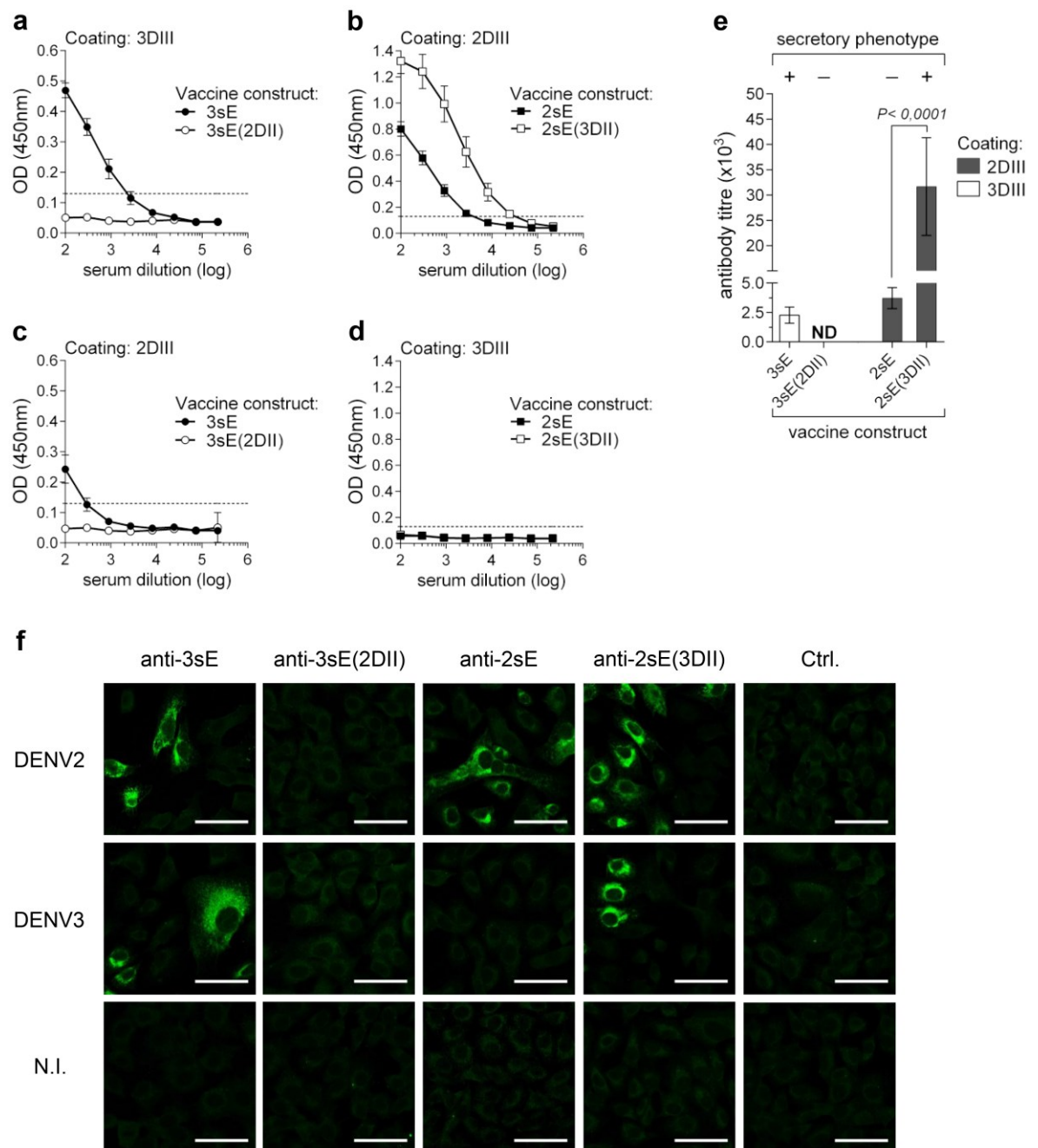


Figure 41. Effect of antigen secretion on antibody responses upon DNA-vaccination in mice. ELISA measurement of anti-DIII antibodies in sera from mice gene gun-immunised with secretory and poor-secretory constructs expressing 3DIII (**a**) or 2DIII (**b**) on plates coated with the corresponding DIII antigen. Cross-reactivity of 3DIII-based sera on 2DIII (**c**) and 2DIII-based sera on 3DIII (**d**) is shown. **e** Homologous anti-DIII antibody titres obtained from all the constructs used for vaccination (for all antigens, $n=6$), data is represented as mean \pm s.d. Statistical comparison between the antibody titres of animals vaccinated with the constructs encoding 2DIII is shown ($t=7,061$ $df=10$), ND indicates antibody titres below the cut-off value at 1/100 dilution. **f** Immunofluorescence of DENV2 and DENV3-infected Vero cells reacted with sera from immunised animals as indicated. Pre-immune (Ctrl.) sera and non-infected cells (N.I.) were used as controls. Bar represents 50 μm . (Adapted from reference 439).

We then used the plaque reduction neutralisation test (PRNT₅₀) on infected Vero cells to measure the capacity of the different sera to neutralise DENV2 and DENV3. Sera from mice vaccinated with the secretory 2sE(3DII) chimera were able to efficiently neutralise

DENV2 and, to a much lower extent, DENV3 (Fig. 42a). As expected from the antibody titres, 2sE-induced antibodies showed limited neutralising capacity towards DENV2, and no activity against DENV3 (Fig. 42b). On the other hand, antibodies from animals immunised with 3sE neutralised DENV3 but not DENV2 (Fig. 42c). In agreement with the ELISA analysis, responses with the poor-secretory 3sE(2DII) chimera did not show any neutralisation activity (Fig. 42d). PRNT₅₀ titres towards the homologous DIII DENV serotype are shown in Fig. 42e.

We then compared the properties of the anti-2DII antibodies obtained with 2sE and 2sE(3DII). Surprisingly, although both immune responses mainly recognised conformational epitopes on 2DIII (Fig. 42f), the avidity of 2sE(3DII)-derived antibodies was significantly higher than those obtained from 2sE (Fig. 42g).

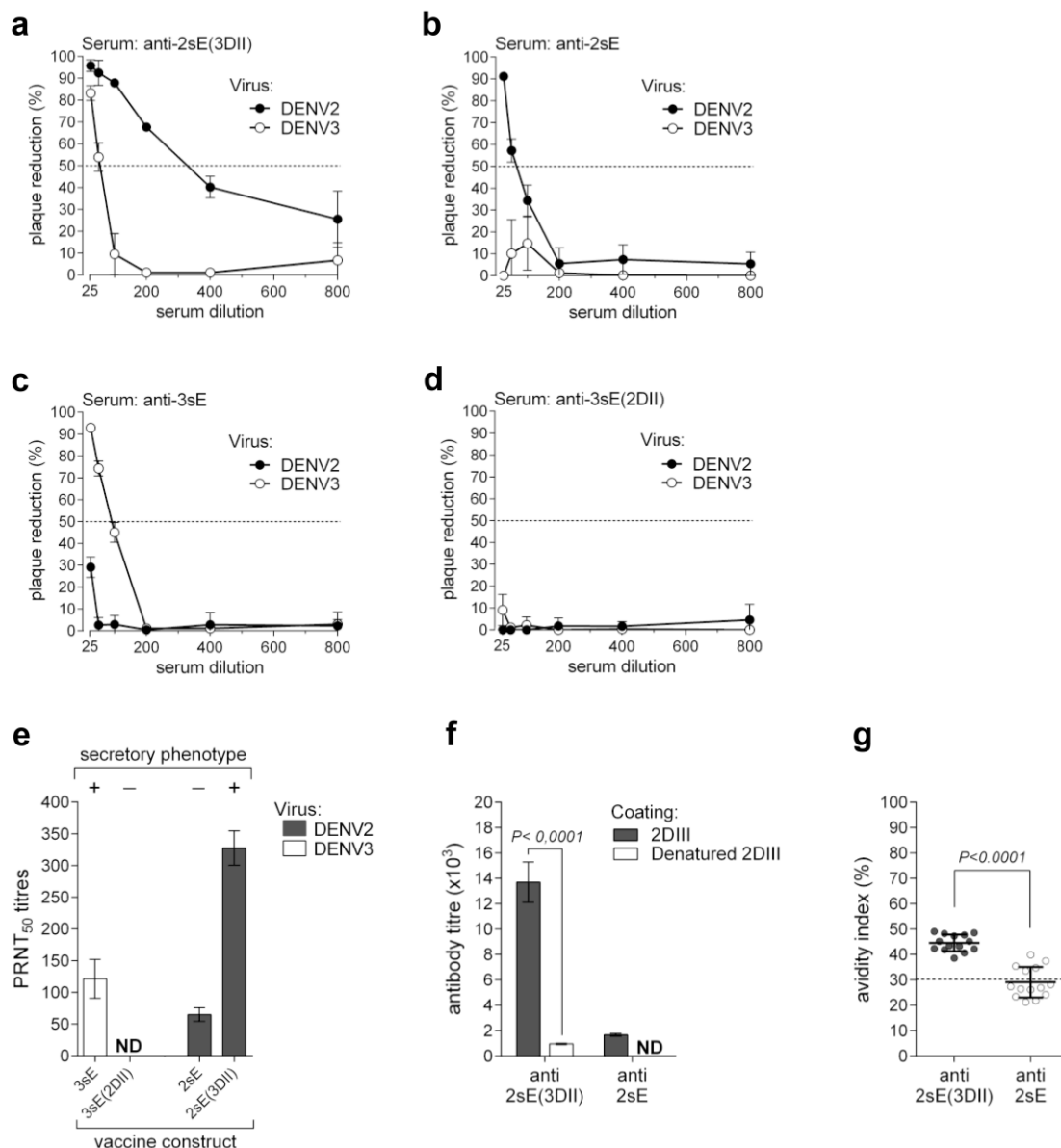


Figure 42. Virus-neutralising activity of secretory and poor-secretory antigens. Plaque reduction neutralisation test (PRNT) on DENV2 (filled symbols) and DENV3 (open symbols) using pooled sera from animals immunised with 2sE(3DII) (a), 2sE (b), 3sE (c) and 3sE(2DII) (d) constructs (in all cases, n=4 for each

virus). **(e)** PRNT₅₀ titres of sera from the indicated secretory and non-secretory constructs on the homologous DENV serotype. ND indicates a PRNT₅₀ titre below 25. **(f)** ELISA performed on equal amounts of native or denatured biotinylated 2DIII, captured on avidin-coated plates and reacted with sera from the indicated constructs (in all cases, n=3). Statistical comparison of the anti-2sE(3DIII) reactivity on native and denatured antigens is shown (t=13,90 df=4). ND indicates an antibody titre below the cut-off value. **(g)** Avidity index of sera derived from animals immunised with 2sE (n=14) or 2sE(3DII) (n=14) determined on native 2DIII (t=8,436 df=26). In all cases, data is represented as mean±s.d. (Adapted from reference 439).

1.3. DISCUSSION

As described before, studies regarding the immune response against flaviviruses suggest that antibodies directed against the E glycoprotein have stronger type-specific neutralisation capacity and represent a lower risk to induce heterotypic ADE when compared to antibodies towards other viral proteins³⁴². In addition, studies on the nature of the epitopes recognised by anti-E antibodies have significantly increased the understanding of interactions between flaviviruses and the immune system, giving valuable tools to develop more efficient vaccine candidates, especially in the case of DENV and ZIKV³¹⁰.

Most of the mAbs with potent neutralising capacity against DENV have been mapped to DIII^{91,92}. Paradoxically, anti-DIII antibodies have almost no effect in the overall immune response against dengue, since the immune response is mainly directed against epitopes on the surface of DI/DII, especially towards epitopes surrounding the FL and the hinge region^{271,273}. However, anti-DI/DII antibodies, which are neutralising only at high concentrations²⁹⁶, have been shown to be highly cross-reactive among the different DENV serotypes²⁷⁹ and ZIKV^{324,444}, which increases the risk of ADE. Recent evidence derived from the study of mAbs isolated from infected patients indicates that conformational epitopes, generated as a result of quaternary interactions between E monomers on the viral surface, are important drivers of the neutralising response after infection²⁷⁹.

We performed a detailed biochemical and structural analysis of the expression, folding, dimerisation and secretion of E ectodomains from DENV serotypes and ZIKV in mammalian cells, with the objective of understanding their secretory properties and implement them in the design of DNA-based vaccines with improved immunogenicity. This is particularly relevant given the importance of conformational epitopes to induce effective immunity against flaviviruses, and the fact that E protein is the main antigenic component of most DENV vaccine candidates^{310,445}.

As shown in our results, sE proteins obtained from the four DENV serotypes have different secretory properties in mammalian cells. While sE proteins from DENV1 and DENV2 showed impaired secretion, DENV3 and DENV4 sE constructs were efficiently

secreted following transfection as evidenced by the presence of the proteins in the culture supernatants. Since expression, demonstrated by the detection of the protein in the cell extracts, was not compromised for any of the constructs, we suspected that 1sE and 2sE were retained in the intracellular compartment due to improper folding.

Based on the primary structure of DENV E proteins and the presence of the poorly structured linker between DI and DIII⁹⁶, we reasoned that sE folding should occur in two co-translational steps: the 3 segments encoding DI and the 2 segments DII need to fold together and establish close molecular interactions to obtain the properly folded DI/DII⁴⁵, while DIII should be able to fold on its own as it derives from a single aminoacid sequence and has no significant interaction with DI and DII in the context of the monomeric E⁸⁵. By splitting the sE constructs into these two theoretical folding units, we were able to match the secretory phenotype of the whole sE to the corresponding DI/DII. The studies using the chimeric constructs further confirmed the assumptions regarding the involvement of DI/DII in determining the secretory phenotype of sE constructs and allowed us to map the poor-secretory phenotype of DENV1 and DENV2 specifically to DII.

To test if protein misfolding was the underlying reason behind the poor-secretory phenotype of constructs carrying DII from DENV1 and DENV2, we probed DII folding in both cell extracts (for non-secreted proteins) and culture supernatants (for efficiently secreted proteins), with mAb 4G2 which recognises the conformational epitope of the highly-conserved FL⁴⁴⁰. Although the reasoning of assuming proper DII folding only on 4G2 reactivity could be argued, we decided to rely on this specific mAb because a) the FL structure depends on a series of complex interactions between several loops and β -strands throughout DI/DII, which requires the correct folding of both domains, b) it recognises all DENV serotypes⁴⁴⁰, which homogenises the readout of the assay and enables comparisons among the different constructs, and c) proper FL structure is instrumental for E dimerisation⁸⁸.

Even though we traced the secretory phenotype of E ectodomains to DII, there is a significantly high identity when comparing DI/DII aminoacid sequences from all four DENV serotypes, regardless of the secretory phenotype. Indeed, the high homogeneity on this portion of E is heavily reflected on the cross-reactivity of the antibodies elicited against DI/DII epitopes²⁷⁹. For example, the sE sequences from DENV2 (poor-secretory) and DENV3 (secretory) share a 71% and 75% identity on DI and DII, respectively.

Thus, a clear indication to explain the differences in folding and secretion, and their direct association with DII, was not apparent from our analyses of the protein sequences. This suggests that the observed phenotypes could be the result of the collective contribution of the small differences distributed throughout the domain. It is also possible that the secretory phenotypes here described are not representative of each DENV serotype but are, instead, dependent on the viral strain; however, as shown in Fig. 43, this

is unlikely since the E amino acid sequences are rather conserved within each serotype with no considerable differences when comparing the diversity found among the different domains.

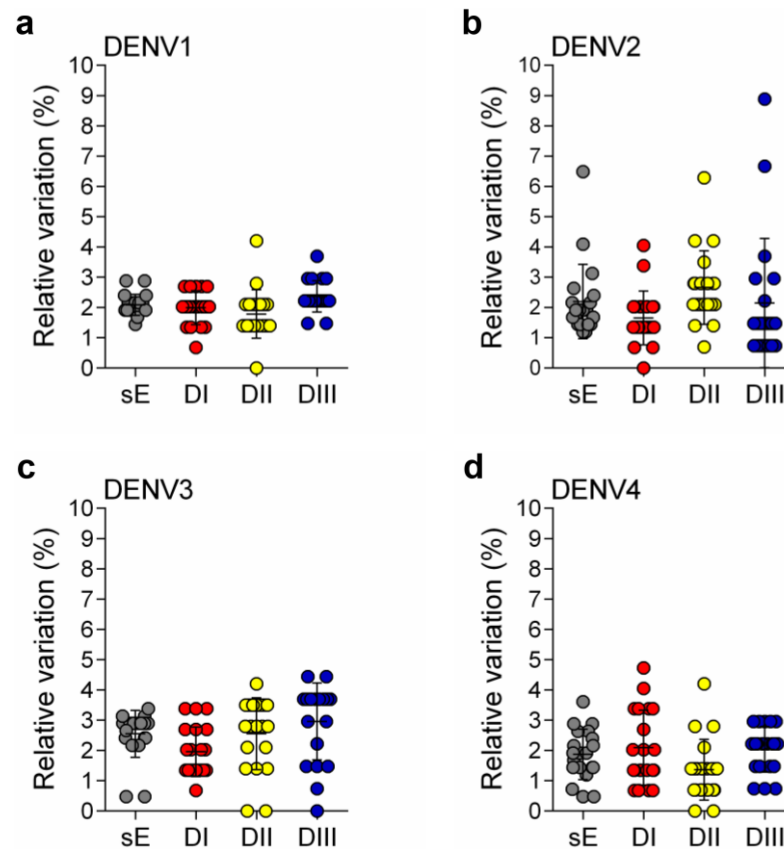


Figure 43. Relative sE amino acid diversity within DENV serotypes. Each of the strains used were compared to 20 randomly selected sE sequences of the corresponding serotype (UniProtKB database, GeneBank accession numbers are shown) and the relative amino acid differences plotted to show variations for the whole E ectodomain (sE, grey) and DI (red), DII (yellow) and DIII (blue). **a)** DENV1 Nauru strain (U88535.1) compared to sequences AF311956.1, D00502.1, D00501.1, M87512.1, AY620953.1, AY620952.1, AAT39550.1, AAT39549.1, AAT39548.1, AAT39547.1, AAT39546.1, AAT40237.1, AAT40236.1, AAT39427.1, AAT37503.1, AAT12783.1, AAT01283.1, AAT00448.1, AAT00447.1, BAC77219.1. **b)** DENV2 New Guinea C strain (AF038403) compared to sequences AEX97780.1, AEX97778.1, AEX97787.1, AAA42942.1, AAA42962.1, ABA61184.1, CAA33474.1, CAA33284.1, BAA00254.1, CAA38217.1, BAA01389.1, AAA73185.1, AAA73186.1, AAA73471.1, AAA42952.1, AAA42954.1, AAA42957.1, AAA42960.1, AAA42951.1, AAD32963.1. **c)** DENV3 H87 strain (M93130) compared to sequences ADF55927.1, ADF55928.1, ADF55929.1, ADF55930.1, ADF55933.1, ADF55936.1, ADF55937.1, ADF55938.1, ADF55941.1, AEW25108.1, AFI71754.1, AFI71773.1, AAV34603.1, AAT75224.1, AAM51538.1, AAM51537.1, BAC77233.1, AAN61123.1, AAN16079.1, AAK01920.1. **d)** DENV4 Dominica strain (AF326573) compared to sequences ABO27186.1, ACC68752.1, ACY01670.1, ADJ18331.1, AEJ89905.1, AER00188.1, AEW24968.1, AEV66313.1, AFI71472.1, AFW15987.1, AFZ40097.1, AAU89377.1, AAU89375.1, AAX48017.1, AAV49746.1, AAV31422.1, AAU89343.1, BAC77238.1, AGH68519.1, AGH68518.1. (Adapted from reference 439).

The recent description of E dimer-dependent quaternary epitopes located on a serotype-invariant site at the E-dimer interface, has significantly impacted the study of

antibody responses against flaviviruses²⁸⁴. Even though the high neutralising capacity of antibodies against complex quaternary epitopes has been previously studied^{278,281,285}, the description of EDE epitopes demonstrate the existence of conserved antigenic determinants that are capable of inducing strong cross-neutralising antibodies against all four DENV serotypes and ZIKV^{286,287,289}, thus opening the possibility for the development of a single universal vaccine. However, the isolation of the precise epitopes outside of the viral surface is a complex, and yet unresolved task that has prevented their implementation into new vaccine formulations. These findings also highlight the importance of ensuring proper E folding and dimerisation in E-based immunogens, in particular for genetic vaccines. In addition, it has been shown that antigens presented in a dimeric structure are able to stimulate immune cell receptors more efficiently, which could lead to stronger antibody responses⁴⁴⁶.

We exploited the parallelism between the E protein cell-surface display and E proteins on flaviviruses to develop an assay that allowed us to study not only the conformation of secreted proteins using highly efficient tools like flow cytometry, but also the interaction between distinct E monomers by cell-surface retention and differential tagging as discrimination parameters. As demonstrated by our data, by replacing the stem-anchor regions of E with the cytosolic and transmembrane domains of the human MHC-I α , we successfully retained the protein on the cell membrane without any apparent alteration to its maturation process within the secretory pathway. Stable interactions between E monomers were then proved by anti-SV5 detection of the efficiently secreted 3sE-SV5 when co-transfected with the membrane-bound homolog, and was also confirmed when analysing sE-DI/DII interactions. However, this strategy was restricted to DENV3 since cells transfected with the secretory sE constructs of the other serotypes and ZIKV were partially retained on the cell membrane and (m)DI/DII constructs were not expressed.

To circumvent this, we developed an alternative approach based on the enzymatic biotinylation of proteins. By co-expression with the *Escherichia coli* derived biotin ligase BirA engineered to target the ER lumen, the system offers the possibility of specifically labelling proteins tagged with the BAP sequence⁴²⁶, a 15aa long peptide with a single lysine residue that serves as biotin acceptor. As previously demonstrated by others, this labelling process is highly efficient, specific and stable, and results in a mono-biotinylated product⁴²⁷, which means that signal detected following treatment with HRP-linked streptavidin are independent from the size or composition of the protein (as opposed to chemical biotinylation) and are directly proportional to the amount of biotinylated-protein detected. Similar to the membrane retention of 3sE-SV5, by combining co-transfection of SV5- and BAP-tagged constructs with anti-SV5 immunoprecipitation, this approach allowed us to detect protein interactions by co-immunoprecipitation of the biotinylated

partner. Relative quantification of the amount of co-immunoprecipitated protein showed that approximately 20% of the secretory sE-BAP protein from DENV3 and DENV4 were able to form stable dimers with their SV5-tagged counterparts, in agreement with reports indicating that sE in solution is mainly present as a monomer²⁸⁴. Interestingly, although it has been described that the majority of the intermolecular interactions involved in sE dimerisation take place on the inner face of DII, our results show that dimer stability is heavily dependent on DIII, as secreted sE-DI/DII hetero-dimers were less abundant than sE-sE homo-dimers, and those between DI/DII completely absent. This role of DIII in dimer stabilisation has been previously suggested^{77,447}.

Since the stability of sE dimers was limited, we introduced an Ala to Cys mutation in the inner surface of the E monomer to covalently stabilise the dimeric structure of sE proteins and study their biochemical properties. The side chain of the inserted cysteine points outwards from the alpha helix B (α B) located on the inner surface of DII, directly facing itself on the opposing monomer once the antiparallel configuration of the dimer is in place, which resulted in dimers covalently stabilised by a disulphide bond.

The data obtained with covalently stabilised dimers further confirm our initial observations regarding the temperature-dependent secretion and folding of 2DI/DII, and indicate that incubation at lower temperatures is important not only for the secretion of stable E dimers, but also for proper folding of E. This is not the first observation regarding the effect of temperature on the overall structure of dengue; recent data showed that dengue virions suffer structural reconfiguration at temperatures above 33°C^{50,51}. At this temperature, the viral envelope expands, increasing the exposure of E which would theoretically improve viral binding to host cells^{48,51}. The data presented here represents, to our knowledge, the first report extending this temperature-dependent behaviour to the sE forms of DENV and ZIKV, especially to sE-cvD proteins, since their secretion was significantly improved with the 28°C transfection protocol. This is in agreement with reports describing enhanced production and stability of DENV and ZIKV virions at relatively low temperatures⁴⁴⁸.

Our data indicates that besides proper folding, secretion of recombinant E proteins from transfected cells depends also on their capacity to dimerise, and that relatively stable dimers are required for successful transit through the ER and the secretory pathway. For DENV3 and DENV4, secretion of sE proteins was observed for both wt and cvD forms regardless of the temperature conditions, suggesting higher stability of dimers and therefore compatibility with secretion; while for DENV1 and ZIKV, induction of proper folding at 28°C was enough to achieve efficient secretion of the recombinant wt protein. In contrast, although our 4G2 analysis showed that proper folding of DENV2 sE is present at 28°C, efficient secretion was only possible in presence of the Ala-Cys mutation, strongly suggesting that wt dimers are relatively unstable within the intracellular secretory

compartment and are only allowed to traffic when covalently stabilised. In fact, recent structural data obtained during cryo-EM analysis of mAb 2D22 on the viral surface of DENV2, showed that this virus is significantly more dynamic when compared to the other DENV serotypes²⁸⁵, which might explain the low stability of the sE-wt dimer and why the disulphide bond was required to achieve secretion.

Following the same approach as before, mAb-based structural analysis of membrane-bound sE-cvD by cytofluorimetry, or biotinylated-dimers captured on avidin-coated plates by ELISA, confirmed that sE-cvD complexes presented proper folding of their monomeric-structural domains, while displaying quaternary epitopes associated with E dimeric configuration on the viral surface. Moreover, the membrane display experiments allowed us to further describe the role of temperature in proper folding of E. Contrary to the analysis by western blot and ELISA, and as revealed by the positive reactivity of dimer-specific mAbs, sE-wt anchoring to the cell membrane stabilised E dimeric conformations probably by keeping the monomers in close proximity after secretion, a close representation of the dynamics that could take place on the viral surface. Under these conditions (m)sE-wt constructs showed the same temperature-dependent secretory behaviour and structure as (m)sE-cvD constructs. These results demonstrate the structural equivalency between the dimers formed by sE-wt and sE-cvD proteins, and prove that the Ala-Cys mutation stabilises the naturally occurring interactions between E monomers.

The level of scrutiny with which we were able to analyse the structural properties of sE-wt and sE-cvD constructs was achieved by incorporating the use of mAbs that target not only specific DENV serotypes, but more importantly, epitopes present at different levels of structural complexity: as previously mentioned, mAbs 4G2 (FL on DII⁴⁴⁰), 4E5A (DIII⁴⁴²) and 1F4 (DI and hinge region of DENV1, but only on the viral surface²⁸²) recognise epitopes restricted to a single structural domain of E; EDE1-C8, EDE1-C10, EDE2-B7²⁸⁴ and 2D22²⁸⁵, recognise epitopes restricted to E dimers while 5J7 docks to an epitope that requires higher order arrangements of DENV3 E dimers²⁸¹. Our data on ELISA and cytofluorimetry assays proves that these epitopes are present in the analysed proteins. Noteworthy, 5J7 reactivity on (m)3sE-cvD constructs indicates that membrane-bound proteins are able to replicate structural arrangements beyond the complexity of E dimers. When compared against (m)3sE-wt constructs (that were efficiently recognised by dimer-specific mAbs, but not by 5J7), data suggest that proper epitope conformation between un-bound proteins, is imposed upon binding of the antibody that holds the interacting proteins into position, stabilising the dimeric conformation in sE-wt protein in a similar way as the disulphide bond holds sE-cvD monomers together. This kind of antibody-mediated stabilisation was also described during structural mapping of EDE

antibodies where interaction with the Fab or single-chain Fv of the corresponding antibodies shifted the equilibrium of sE proteins towards the dimer conformation²⁸⁴.

Since 5J7 epitope requires the simultaneous interaction of 3 independent E monomers in two adjacent E dimers of the raft conformation on the viral surface²⁸¹, it is possible that the antibody is not able to achieve that level of stability with the (m)sE-wt proteins while the covalent stabilisation of cvD mutants facilitates the interaction as the antibody only requires to hold two independent units in position instead of three. A similar mechanism may be involved with the ability of dimer specific antibodies to recognise their respective epitopes in (m)wt proteins and explain the discrepancies observed between ELISA and cytofluorimetry profiles, since the fluidity of the cell membrane permits transient protein interactions due to horizontal mobility on the cell surface, while this would not be possible for the molecules immobilised on the ELISA plates.

In addition, regarding the activity of mAb 1F4, which recognises DENV1 E protein on the surface of the viral particle but not as a recombinant protein secreted from insect cells²⁸², there are two possible scenarios: either a) mAb 1F4 indeed recognises an E conformation that is restricted to the viral surface, or b) the recognised epitope is dependent on the dimeric conformation of E, rather than one exclusively present on the virus, but was unresolvable due to the lack of E dimers outside of the viral particle context. Taking into consideration the reactivity profile of mAb 5J7, and the positive reactivity of 1F4 to (m)1sE-wt and (m)1sE-cvD proteins, our data supports the latter; even though we were unable to do a definitive confirmation due to the inability to test DENV1 sE-cvD proteins in ELISA. Still, the possibility of achieving higher levels of resolution when analysing the structural complexity of epitopes, highlights the potential of our analytic platform.

In infected cells, proper E folding and assembly could rely on the interactions with other viral proteins and host factors. For instance, the stem-anchor domains have been shown to negatively affect the production and secretion of sE proteins from transfected cells⁴⁴⁹ while they are obviously incorporated in virions. As shown in Fig 44 we confirmed the detrimental effect of the stem region on production and secretion of 3sE by using constructs encoding the sE alone, or fused to the first alpha helix (sE-H1), or the full stem region (sE-stem), at C-terminus.

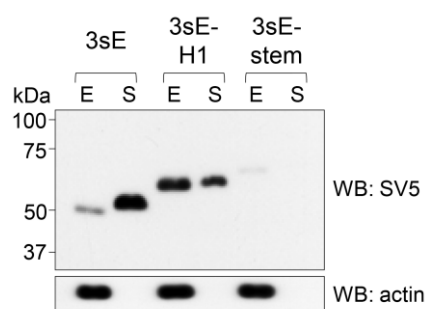


Figure 44. Effect of the stem region on production and secretion of sE proteins from mammalian cells.

WB analysis of cellular extracts (E) and culture supernatants (S) of HEK-293T cells transfected with constructs encoding DENV3 sE protein without the stem-anchor region (3sE), with the first 16 aminoacids of the stem (3sE-H1) and the full stem (3sE-stem). Anti-actin was used as loading control.

In addition, the role of viral PrM in ensuring efficient viral release by preventing premature fusion of the nascent particles during their transit in the secretory pathway, has been addressed by previous studies⁷⁷. Although it has been suggested that PrM could also have a chaperone-like function in assisting E proper folding, the experimental evidence regarding this property is less convincing, with some studies showing that properly folded sE secretion from transfected cells is dependent on PrM co-expression^{79,450,451}, while others have shown secretion of properly folded sE on its own^{102,452}. More recently, reports evaluating ZIKV E protein candidates for genetic vaccination observed poor immunological response, in both cases this was explained by claiming poor protein stability because of the lack of PrM co-expression, even though experimental data supporting these assumptions was not shown^{453,454}. In contrast, a recent DNA vaccine candidate against ZIKV, showed immune stimulation using a plasmid encoding only the MsE region of the viral polyprotein, suggesting that the inclusion of Pr was not necessary⁴⁵⁵. Our results clearly show that, surprisingly, temperature, but not PrM co-expression, is essential for E dimerisation, folding and efficient secretion from mammalian cells. This is particularly relevant when considering E-based subunit and genetic vaccines against DENV or ZIKV including PrM on the basis of ensuring proper antigen production, since it has been demonstrated that anti-Pr antibodies have almost non-existent neutralising capacity and mostly favour viral infection through ADE²⁹¹. Given that we showed successful expression of complex quaternary epitopes regardless of PrM co-expression, these results provide further evidence supporting PrM exclusion from vaccine formulations.

As shown by the experiments with pseudoviruses, the Zika virus E-cvD construct (ZE-cvD) was able to package the WNV-rep into secreted particles, which showed all the E protein present as covalently-bonded dimers. Supporting the effect of temperature on E folding and dimerisation, and in line with the behaviour of sE-cvD protein, packaging of particles with ZE-cvD was significantly higher at 28°C. Overall, the results confirm that the

covalent dimers not only have the proper antiparallel conformation, but are also compatible with the complex arrangements required to assemble the viral particle. The assembly and secretion of these pseudoviral particles indicate that E dimerisation occurs shortly after translation, and that transport and secretion of full particles as the covalently stabilised dimers is permissive. In addition, it also indicates that the “spiky” immature intermediates observed in the ER lumen are not strictly required for particles to traffic through the secretory pathway. Yet, they may reflect the highly dynamic structures of E needed to fulfil the viral life cycle⁴⁵⁶.

As expected, these particles were not infectious as E trimerisation, and thus the fusion-mediated escape of the viral RNA from the early endosome, was inhibited by the disulphide bond. This supports the idea that viral neutralisation can be obtained by interfering with the dynamics of viral E, a mechanism that has been proposed to explain the strong neutralising capacity of antibodies targeting complex quaternary epitopes^{285,288,457}. In this mechanism, docking of the antibody to its epitope locks E structure into a fixed configuration thus preventing the trimerisation process⁴⁵⁷. A similar mechanism has also been proposed for antibodies targeting the hinge region³¹⁰, highlighting the importance of E conformational changes.

The description of hetero-dimers between sE proteins from different viruses was surprising. The alternative use of SV5- and BAP-tagged proteins in the context of WB experiments, allowed us to clearly detect the presence of hetero-typical complexes, a finding that was confirmed using an adapted version of the membrane display assay described previously. Interestingly, the results were successfully replicated with the (m)sE-wt constructs, which indicate that these interactions occur naturally. The same conclusions were drawn from the production of mosaic pseudoviruses co-expressing E proteins from ZIKV and DENV2.

The mosaic nature was demonstrated by ADE experiments on K562 cells with anti-DENV2 specific antibodies. Although at present we lack the means to describe the structural characteristics of these mosaic particles, our data indicates that they can exist as infective particles. It is difficult, however, to speculate on the true impact (if any) of these hypothetical particles during the infection process, since single-cell flaviviral co-infection has not been reported.

Collectively, these data are in line with observations that the residues involved in E dimerisation are conserved across the flaviviruses, explaining not only the cross-neutralising nature of antibodies against quaternary epitopes^{284,288,457}, but also allowing for heterotypic interactions without disrupting viral infectivity, as shown here. In fact, a recent study compared the sE sequences from different flaviviruses and showed that the level of

aminoacid identity of the α B helix is only matched by that of the FL (Fig. 45), highlighting the importance of this region for the dimer structure of E and the viral fitness⁴⁵⁸.

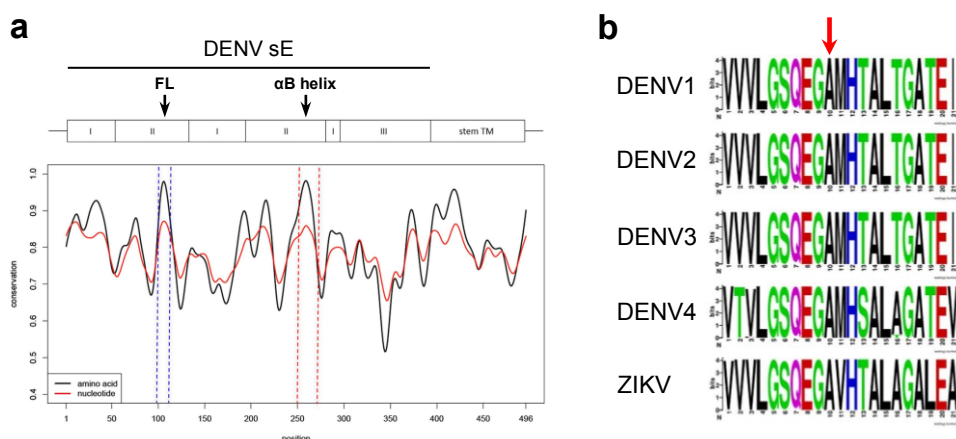


Figure 45. Conservation of α B helix among DENV serotypes and ZIKV. a) Conservation profile of the nucleotide (red line) and aminoacid (black line) sequences of 480 coding sequences; a conservation value of 1 represents the highest conservation possible. A schematic representation of the primary structure of E shows the relative position of the corresponding residues (and bases) on the protein, the FL and α B helix location are indicated. b) WebLogo schematic showing the aminoacid composition of the E250-270 region (which includes the α B helix) for each DENV serotypes and ZIKV, the highly conserved alanine involved in the cvD mutations is indicated (red arrow). Polar (green), neutral (purple), basic (blue), hydrophobic (black) and acidic (red) aminoacids are shown. (Adapted from reference 458)

Limited understanding of viral pathogenesis has been an important hurdle in developing treatments against viruses like DENV and ZIKV. Although it has been shown that the antibody response is one of the most important mechanisms to neutralise the virus, the specificity required for efficient protection is still unknown³¹⁰. Due to their strong neutralising potential and low ADE risk, antibodies against DIII and complex quaternary epitopes have been singled out by studies of monoclonal antibodies isolated from animal model or immune patients for future vaccine formulations⁴⁴⁴. Together, the ELISA and cytofluorimetry based-assays described in this section, constitute a new virus-free platform to study anti-E antibodies and differentiate their binding properties. Indeed, the use of different E-derived constructs allowed us to discern between mAbs able to bind single E domains, the monomeric sE, sE dimers or even higher order arrangement of dimers without the concerns related to the use of whole viral preparations, and can be implemented to screen for antibodies that target neutralising epitopes.

Given that most of the strongly neutralising antibodies target conformational E epitopes⁶⁴, reproducing the native conformation of this viral protein is pivotal for the development of an efficient vaccine against DENV and ZIKV¹⁰². Several DNA and protein subunit vaccines have been developed based on truncated versions of E obtained after removing the anchor regions of the viral protein^{375,376,407,459}. Strikingly however, only a few of the studies involving DNA vaccination reported the efficiency of production and

secretion of the antigen from transfected cells, and experimental data supporting proper folding of the antigen was also rarely published. In the context of DNA vaccines, a previous study compared the secretion of different full-length and truncated version of DENV1 E constructs⁴⁶⁰. The results revealed that all sE constructs were poorly secreted from transfected cells even when co-expressed with PrM and induced only modest neutralising responses in vaccinated animals. A DNA vaccine candidate encoding 2sE was shown to be poorly secreted from BHK-21 cells and induced low neutralising titres in vaccinated mice⁴⁰⁸, closely resembling the anti-2sE response reported here. While these results are in agreement with our observations on the secretory phenotypes of DENV sE proteins, an AAV-based genetic vaccine candidate recently reported a secretion analysis for sE proteins from all four DENV serotypes indicating that only 1sE was efficiently secreted⁴²². Collectively, these data suggests that sE secretion from transfected cells might also be influenced by other yet undefined factors, but highlight the importance of performing this sort of profiling to evaluate secretory properties of antigen design for genetic vaccines.

After the first clinical trial involving a DNA vaccine against HIV⁴⁰¹, several other DNA vaccine candidates have been developed against a range of infectious agents⁴⁰²⁻⁴⁰⁶. Even though a series of studies have attempted to apply the DNA vaccination approach to dengue^{390,407,408,422,461-467}, only one of these candidates was able to progress to a Phase 1 clinical study using a plasmid coding for PrME of DENV1⁴¹⁰. As mentioned previously, this candidate was reformulated and a new tetravalent vaccine based on this format is now being evaluated in Phase 1 studies³⁴². Although, low immunogenicity has prevented further advances in the field, the different human tests that have been conducted, confirmed the safety of DNA vaccines^{413,414}. In our case, the careful combination of constructs designed to express the same antigenic but with different secretory phenotypes, demonstrate that antigen secretion is indeed a critical aspect to consider when designing an efficient candidate for genetic vaccination. Even though immunogenicity is also a critical aspect to consider, failure to examine and improve the secretory efficiency of encoded antigens may explain the poor performance of the DNA vaccines tested so far against DENV^{460,466,468,469}. Our chimera approach also shows that chimeric constructs could be used to improve the immune response towards specific epitopes that are poorly secreted on their source protein, as the response against 2DIII was significantly improved by modifying the sE in which it was expressed. These modifications not only improved the antibody titres and the neutralising response after vaccination, but also the avidity of the elicited antibodies.

As a corollary, the results presented in this section provide a careful and detailed dissection of the factors regulating sE proteins expression and secretion from mammalian cells, and demonstrate that proper design and evaluation of encoded antigens for DNA

vaccines is crucial to develop strong neutralising responses against DENV, the main focus of the next section of this thesis.

PART II

DEVELOPMENT AND PRELIMINARY EVALUATION OF A DIII-BASED DNA VACCINE AGAINST DENGUE VIRUS

2.1. SYNOPSIS

Developing an effective vaccine against dengue has been one of the highest priorities for researchers in the field. Recent data questioning the effectiveness of the only licensed vaccine available, Sanofi-Pasteur's Dengvaxia®, has revamped the interest for new alternative candidates using next generation approaches like genetic vaccination and properly designed subunit vaccines. However, recent description of immunological cross-reactivity between flaviviruses, especially DENV and ZIKV, has added new hurdles to the already complex task of developing a balanced and effective tetravalent formulation to avoid ADE. With the objective of developing an efficient DNA vaccine against DENV, we designed four DIII-based constructs and evaluated their immunogenicity by gene gun vaccination in mice. Likewise, the results of different tetravalent formulation are also described.

In addition we also evaluated the immunological properties of other E-based genetic constructs, mainly DI/DII and sE, to assess their potential as immunogens in DNA vaccines.

Our results indicate that properly engineered DIII-based constructs are able to drive strong neutralising antibody responses that remain stable for at least a year after vaccination. Moreover, contrary to DI/DII or sE constructs, the antibodies elicited against DIII were highly specific towards DENV and did not promote ADE of other related flaviviruses like ZIKV, WNV and YF, supporting further development of this candidate.

It should be noted that the experiments and results presented in this section were done in close collaboration with Dr. Monica Poggianella (Molecular Immunology Group, ICGEB). Other relevant collaborations are duly acknowledged.

2.2. RESULTS

As in the previous section, some of the data presented in this section were published as a research article in PLOS Neglected Tropical Diseases⁴⁷⁰. Most of the remaining data has been submitted for publication in two additional research articles.

2.2.1. Design and evaluation of a tetravalent DIII-based DNA vaccine.

2.2.1.1. Production and secretion of engineered DIII antigens.

As demonstrated before, DNA vaccines rely heavily on efficient antigen production and secretion from transfected cells for inducing proper B cell activation and strong antibody responses. Similar studies have also concluded that broad antigen expression is required for optimal induction of acquired immunity in the framework of DNA vaccines⁴⁴³.

Fragments encoding the DIII domain of all four DENV serotypes (carrying the nucleotide sequence obtained from viral cDNAs and shown as translated products in Fig. 46a) were cloned into pcDNA3.1(+) vectors with or without the dimerising CH3 domain from the human IgG H-chain (γ CH3) fused at the C-terminus (DIII and DIII- γ CH3 (henceforth referred as DIII- γ), respectively). In addition, a secretion leader peptide (sec) at the N-terminus to ensure translocation into the ER, and the SV5 tag to facilitate detection of the protein were also included (Fig. 46b). The secretory profiles of the encoded proteins in transfected HEK-293T cells are shown in Fig. 46c. In agreement with our previous results, DIII constructs were poorly secreted from transfected cells. However, the addition of the γ CH3 domain significantly enhanced active production and secretion of all DIII- γ constructs. Knowing that efficient antibody response to a DNA vaccine is a corollary of properly secreted antigens, we decided to incorporate the DIII- γ format as the core antigen for our DNA vaccine.

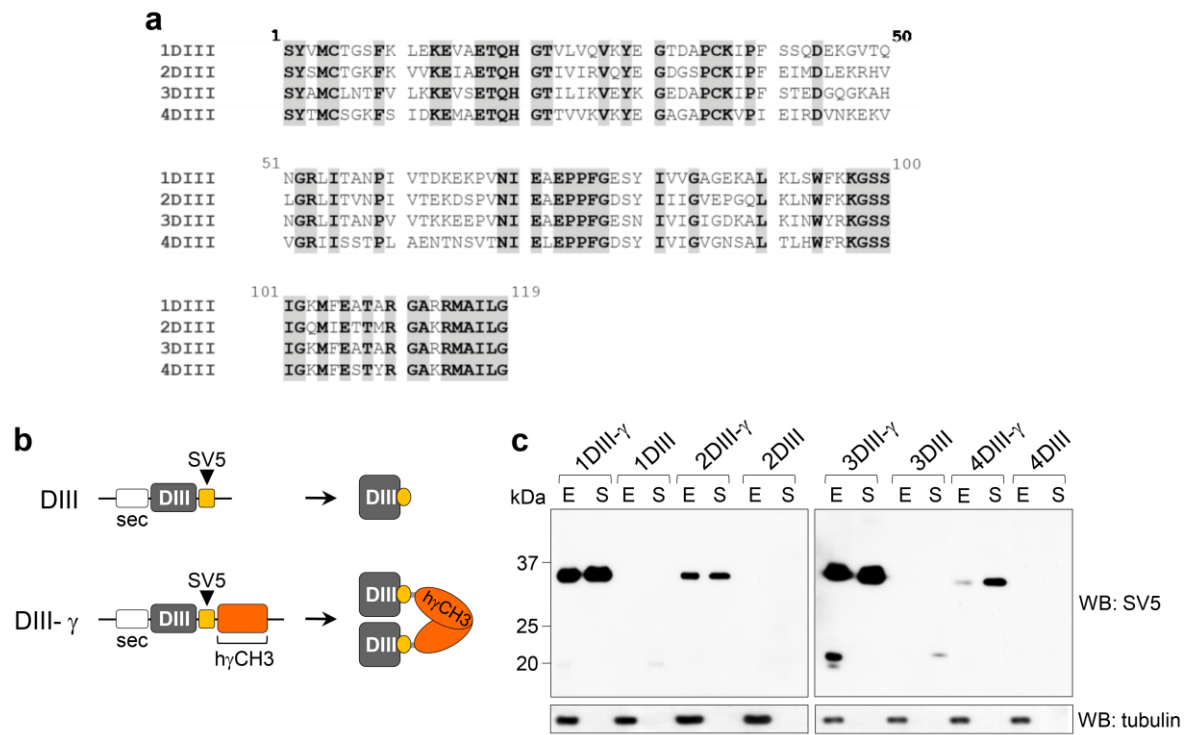


Figure 46. Enhancement of DIII expression and secretion by γ CH3 domain. **a)** Amino acid sequences of the DIII domains from each DENV serotype. Amino acids conserved across the four serotypes are highlighted. **b)** Schematic representation of the plasmids encoding the DIII alone or C-terminally fused to γ CH3 domain. **c)** WB of cell extracts (E) and culture supernatants (S) of HEK-293T cells transfected with the DIII and DIII- γ constructs from all DENV serotypes; anti-tubulin was used as loading control. (Adapted from reference 470).

Another important observation made from the aforementioned experiment was that DIII- γ proteins were not equally obtained in supernatants; notably, the efficiency of secretion for 1DIII- γ was clearly higher, while the amount of 4DIII- γ recovered after transfection was very low (Fig. 47a). With the idea of increasing production in transfected cells, the nucleotide sequences of DENV2, DENV3 and DENV4 DIII- γ plasmids were codon-optimised for mammalian cells. As shown in Fig. 47b, optimised DIII- γ showed enhanced secretion compared to constructs carrying the viral sequence and the amount of protein recovered from transfected cells was comparable for all serotypes.

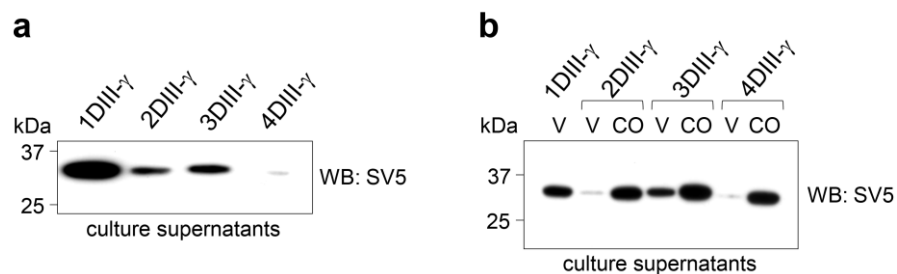


Figure 47. Codon-optimisation increases DIII- γ secretion from transfected cells. **a)** WB of supernatants (S) of HEK-293T cells transfected with equal amounts of the four non-optimised DIII- γ plasmids. **d)** WB of supernatants from cells transfected with DIII- γ constructs with the viral-encoded (V) or the codon-optimised (CO) nucleotide sequences. (Adapted from reference 470).

2.2.1.2. DIII- γ constructs induce strong and highly specific antibody responses in mice.

Experimental groups comprising 10 female Balb/c mice, were DNA-immunised by intradermal biolistic delivery of three 1 μ g doses of plasmids encoding a specific DIII- γ given at 15 days intervals (Days -30, -15 and 0). Mouse sera were collected at days 15 and 30 and analysed for anti-DIII antibodies in conformational ELISA using normalised amounts of *in vivo* mono-biotinylated DIII (for all DENV serotypes) and sE (for efficiently secreted 3sE and 4sE) proteins (produced with the BAP-/BirA system) captured on avidin-coated plates. To avoid interference of antibodies elicited against the γ CH3 domain, the DIII antigens used for ELISA were fused to the human IgE H-chain (ϵ CH4), thus enabling efficient DIII secretion without γ CH3 cross-reactivity. In both DIII and sE antigens for ELISA, the SV5 tag was removed as well.

High titres of homologous anti-DIII antibodies were obtained for all four DENV serotypes (Fig. 48a-d, left panels), and the immune responses elicited with all DIII- γ constructs were comparable among the mice treated within each group (Fig. 48a-d, right panels). In addition, there was no significant difference from sera obtained 15 or 30 days post-vaccination. As shown in Fig. 48e, antibody titres ranged from 19,500, for 1DIII, to 41,300, for 2DIII; which correspond to approximated DIII-specific IgG concentrations of 16 μ g/ml and 35 μ g/ml, respectively (Table 1). Moreover, the anti-DIII antibodies were able to recognise the DIII antigen expressed in the context of the full sE protein, as indicated by the anti-3DIII and anti-4DIII reactivity to both DIII and sE antigens.

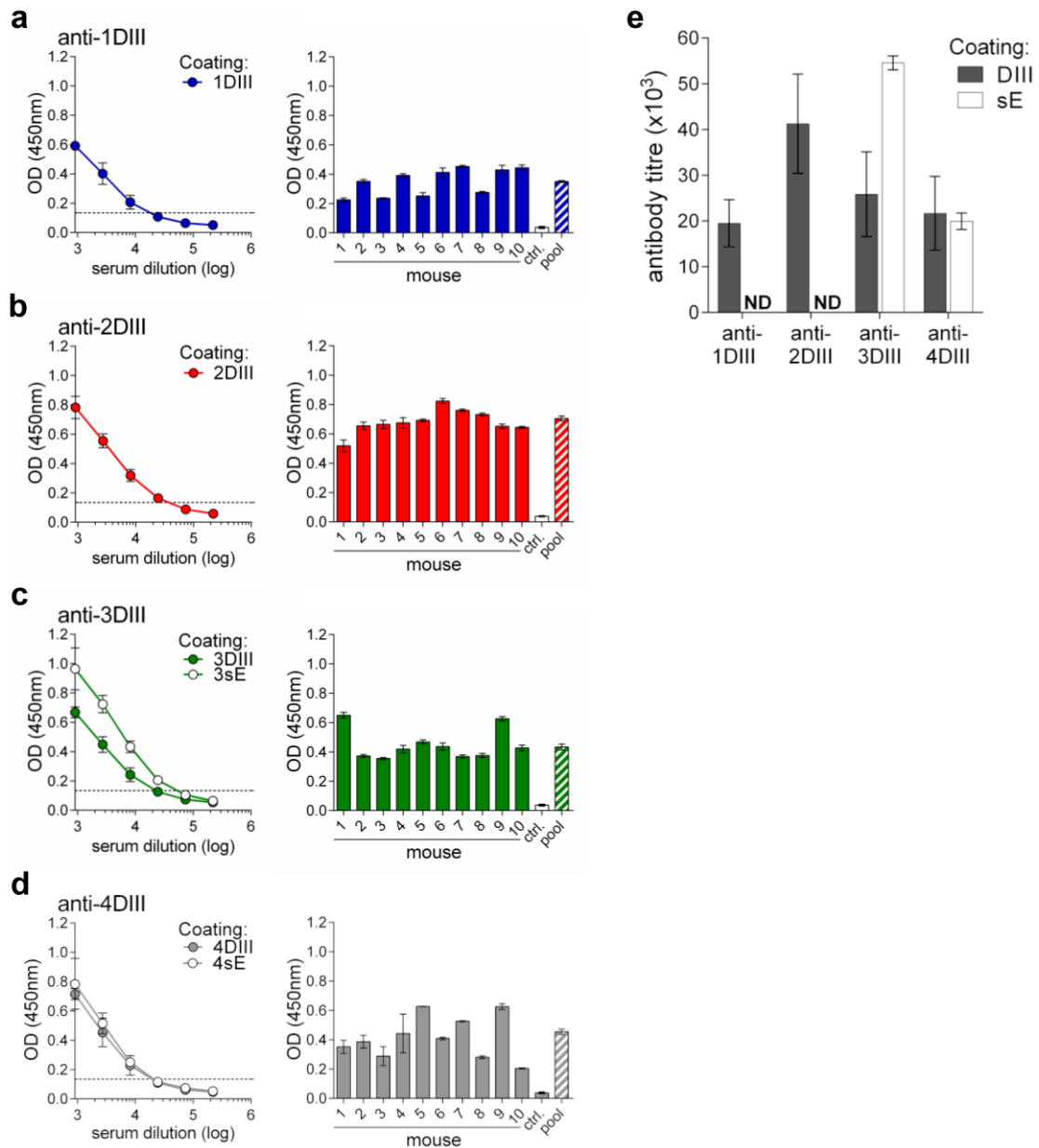


Figure 48. Anti-DIII responses measured in conformational ELISA. **a-d)** ELISA reactivity performed on the homologous DIII antigens for **(a)** anti-1DIII (n=14), **(b)** anti-2DIII (n=14), **(c)** anti-3DIII (n=16) and **(d)** anti-4DIII (n=15) pooled sera from vaccinated animals (left panels) and reactivity of each individual sera compared to the corresponding pool (OD₄₅₀ at a 1:2700 dilution, n=4 for each mice, pooled sera and ctrl.) (Right panels). Ctrl: pre-immune sera. For anti-3DIII and anti-4DIII pooled sera, reactivity against 3sE (n=10) and 4sE (n=6) proteins, respectively, was also included. **e)** Antibody titres of each pooled sera against its homologous DIII antigen; titres determined on the corresponding sE proteins were also included for anti-3DIII and anti-4DIII sera. In all cases, data is represented as mean \pm s.d. (Adapted from reference 470).

Table 1. Estimated homologous anti-DIII concentration in sera from vaccinated mice. Anti-DIII ELISA titres expressed as antibody concentrations, obtained from dilution curves compared to mAb 4G2.

Immunogen	Coating	[Antibody] (μ g/ml)
1DIII- γ	1DIII	16 \pm 3
2DIII- γ	2DIII	35 \pm 9
3DIII- γ	3DIII	21 \pm 3
4DIII- γ	4DIII	20 \pm 1

To evaluate if the anti-DIII antibodies were able to recognise not only the recombinant protein used in ELISA, but also the E protein expressed in virus-infected cells, we performed immunofluorescence assays on Vero cells infected with the different DENV serotypes. As seen in Fig 49a, all anti-DIII sera were able to recognise the homologous E protein in infected cells. This reactivity was further confirmed by ELISA on infective viral particles (Fig. 49b-c). Together, these results suggest that the structure of the DIII, as displayed in the DIII- γ antigen, successfully replicates the structure of the domain in the virus.

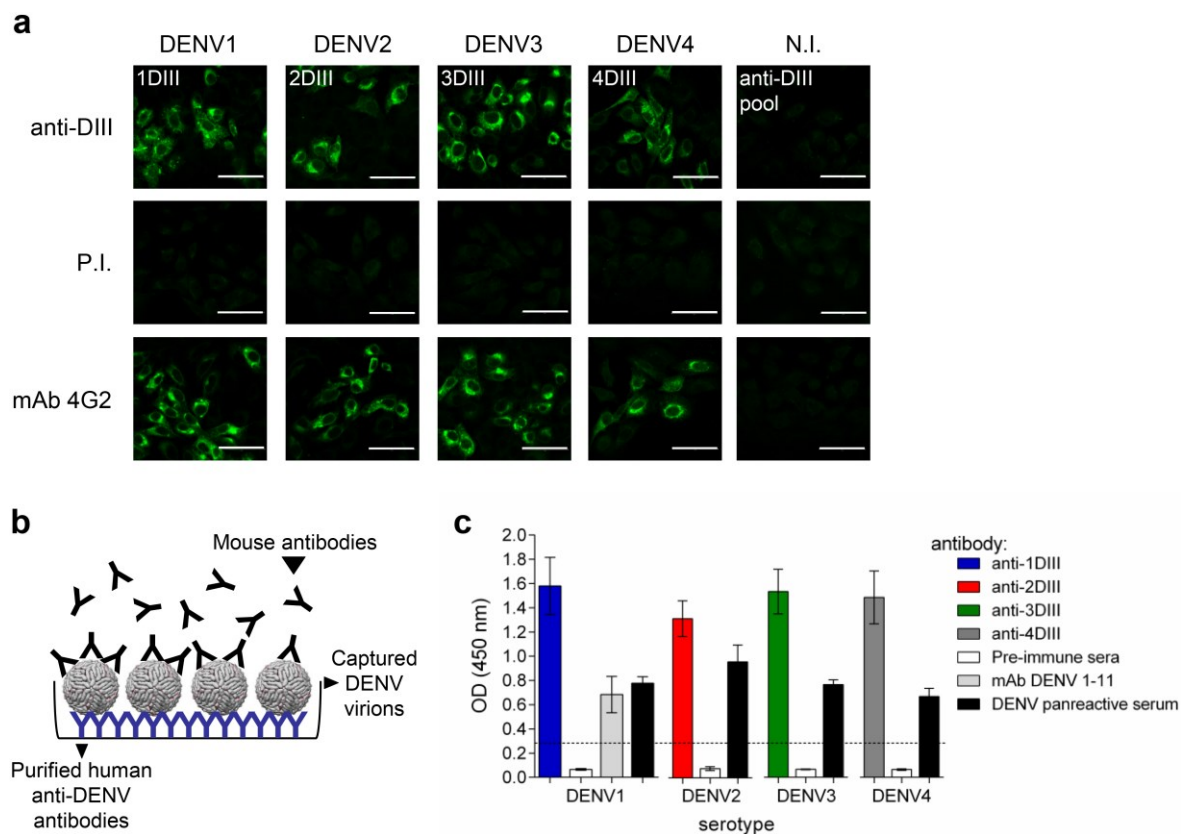


Figure 49. Anti-DIII sera recognise viral E protein. **a)** Immunofluorescence of Vero cells infected with each DENV serotype, reacted with the serotype-specific anti-DIII pools of sera (top row), pre-immune sera (P.I., middle row) and mAb 4G2 (bottom row), were used as negative and positive controls, respectively. Non-infected cells (n.i., rightmost column) were also used as controls. Bars represent 50 μ m. **b)** Schematic representation of the ELISA on infective viral particles. **c)** Homologous anti-DIII pooled sera (diluted to \approx 100 ng/ml) and pre-immune sera were used for ELISA on whole infective viral particles captured with a human serum reactive against all four serotypes. mAb Dengue 1-11 (reactive against DENV1 E, at 1 μ g/ml) and a Dengue pan-reactive serum against all four serotypes were used as positive controls. In all cases, data is represented as mean \pm s.d. (n=4). (Adapted from reference 470).

We then tested the anti-DIII sera against equal amounts of native and denatured DIII antigens in ELISA. As seen in Fig. 50a-b, the four anti-DIII sera lost most of their reactivity when reacted against the denatured protein, indicating that antibodies induced with the DIII- γ antigens recognised conformational epitopes. In addition, avidity studies performed

with the immune sera, which measure the relative strength of antibody binding under stringent dissociating conditions, confirmed that high affinity antibodies were present in a significant concentration (Fig, 50c). The avidity index of mAb 4G2 against 3sE and 4sE was included as a control.

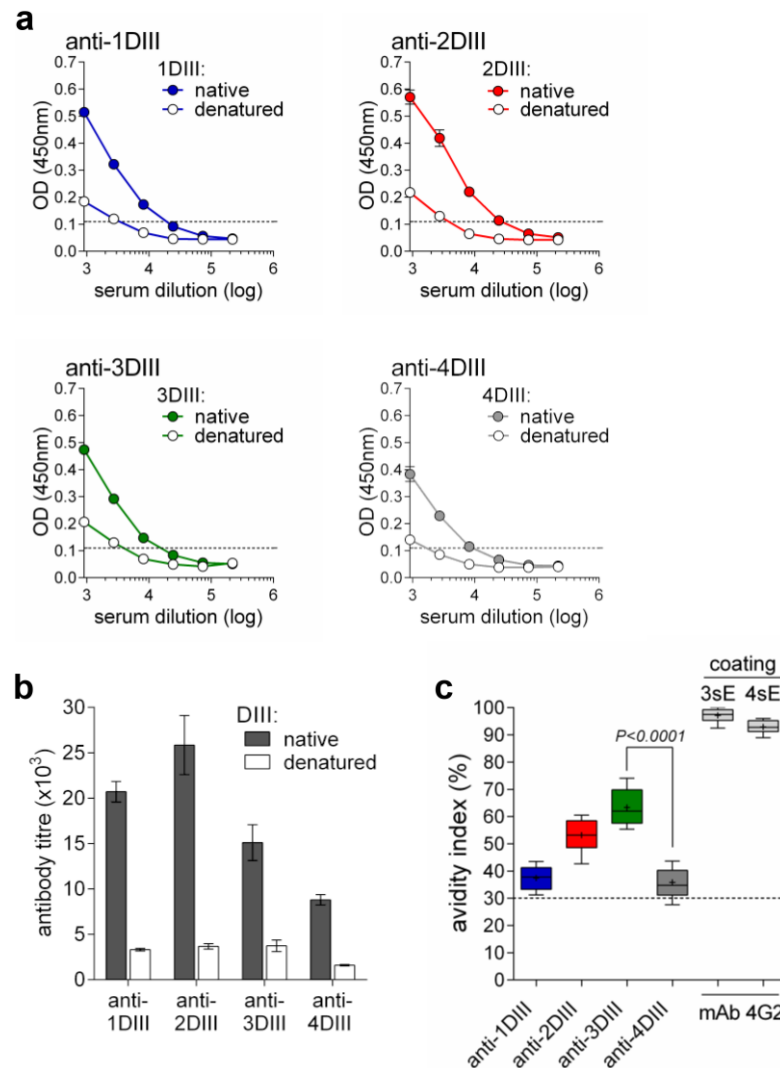


Figure 50. DIII- γ induced antibodies recognise conformational epitopes with high avidity. a) Reactivity of the four anti-DIII sera on equal amounts of native or denatured biotinylated homologous DIII- ϵ CH4 proteins (in all cases, $n=3$). Data is represented as mean \pm s.d. **b)** Antibody titres from the curves shown in (a). Data is represented as mean \pm s.d. **c)** Box and whiskers plot of the avidity index for each anti-DIII sera on the native homologous DIII- ϵ CH4 antigen ($n=14$ for each sera; for the comparison between anti-3DIII and anti-4DIII, $t=12.08$ $df=26$). Avidity index of mAb 4G2 on 3sE ($n=6$) and 4sE ($n=8$) is shown as a control. + indicates the mean value for each group. (Adapted from reference 470).

We then sought to determine the extent of cross-reactivity induced by the DIII- γ vaccines. For this we measured the reactivity of the different anti-DIII sera against 3sE, 4sE and the four DIII antigens (Fig 51a). Although, as expected, each pooled sera showed highest reactivity against the homologous serotype in a conformational ELISA, the level of heterotypic recognition differed among them. As shown by the antibody titres presented in

Fig. 51b, anti-DIII antibodies against DENV1, DENV2 and DENV4 were highly specific, with only limited cross-reactivity to other serotypes. Anti-3DIII antibodies, in contrast, were cross-reactive to 4DIII, 2DIII and 1DIII. The data in Table 2 summarises the cross-reactivity results by showing the titres obtained against heterotypic serotypes as relative values of the homologous response, i.e. expressing the cross-reactive response as a percentage of the antibody titre obtained against the homologous serotype.

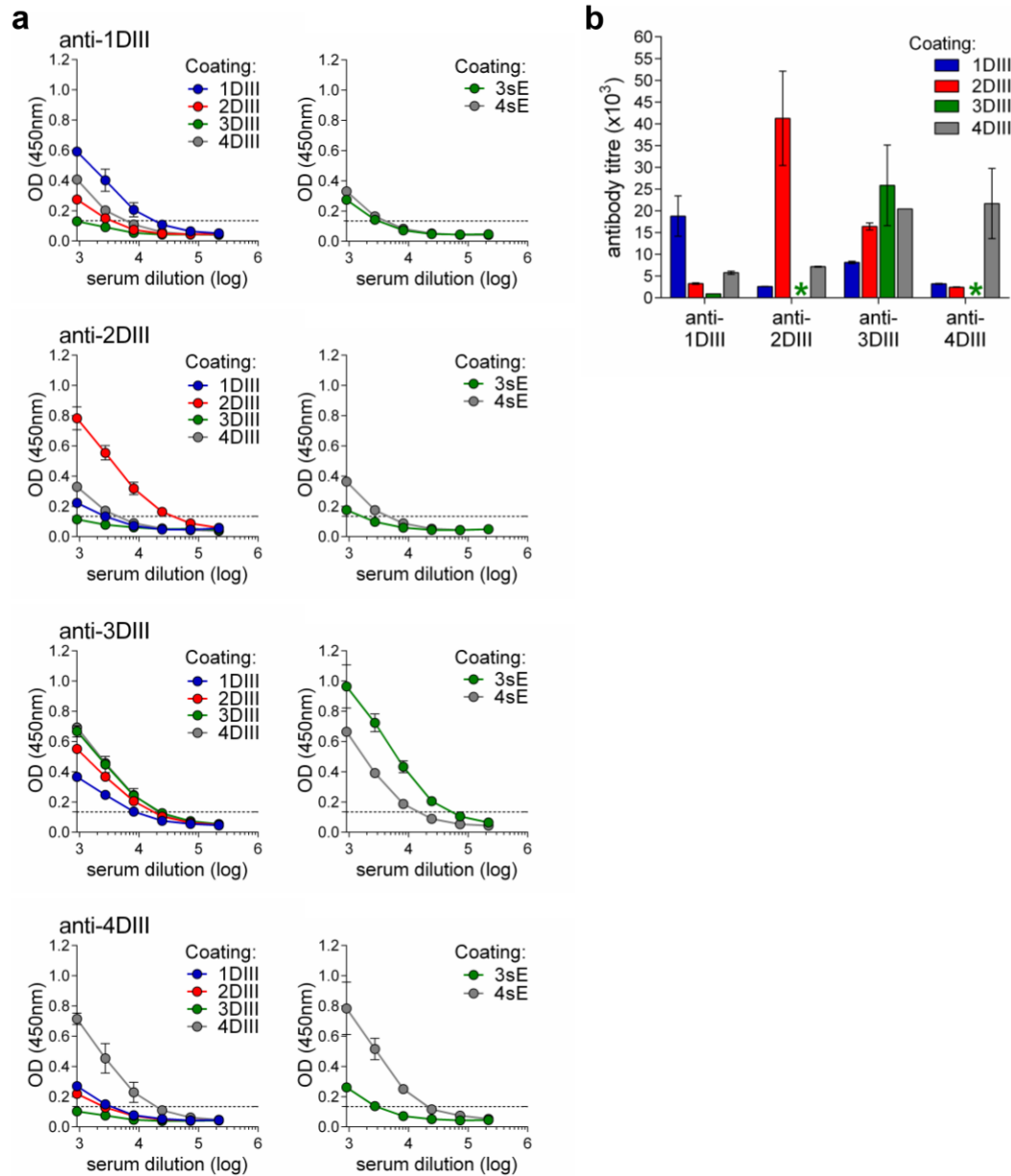


Figure 51. Cross-reactivity profiles of anti-DIII antibodies. **a)** ELISA reactivity of the four different anti-DIII pooled sera on the four DIII- ϵ CH4 (left panels) and on the two secreted sE (3sE and 4sE), (right panels) (In all cases, $n=4$). **b)** Cross-reactive titres from the curves shown in **(a)**. * indicates titre below control (absence of cross-reacting antibodies). Data is represented as mean \pm s.d. (Adapted from reference 470).

Table 2. Relative cross-reactivity of serotype-specific anti-DIII sera. Reactivity of each anti-DIII pooled sera against each all four DIII-εCH4 antigen, expressed as a percentage of the reactivity against the homologous antigen (100%).

Coating	Serum			
	anti-1DIII	anti-2DIII	anti-3DIII	anti-4DIII
1DIII	100	14	43	17
2DIII	8	100	40	6
3DIII	3	ND	100	ND
4DIII	27	33	94	100

ND: cross-reactivity was not detected

2.2.1.3. Antibodies induced by DIII-γ vaccines show neutralising activity.

After determining the level of DIII-specific antibodies induced by DIII-γ vaccines, we tested the neutralising capacity of each serum against the corresponding DENV serotype by means of a plaque reduction neutralisation test (PRNT₅₀) in Vero cells. Fig. 52a shows the PRNT₅₀ curve of each pooled sera against its homologous serotype (Fig. 52a, left panels). The neutralisation titres obtained were representative of the variation found when evaluating individual mice within each group. Although with some differences (particularly in the group immunised with 3DIII-γ), the DIII-γ vaccines induced similar neutralising responses in most vaccinated animals (Fig. 52a, right panels). When comparing the results from each group, we found high neutralising titres against DENV1 (≈300), DENV2 (≈1600) and DENV3 (≈300), while the neutralising response against DENV4 was somewhat weaker (≈65) (Fig. 52b).

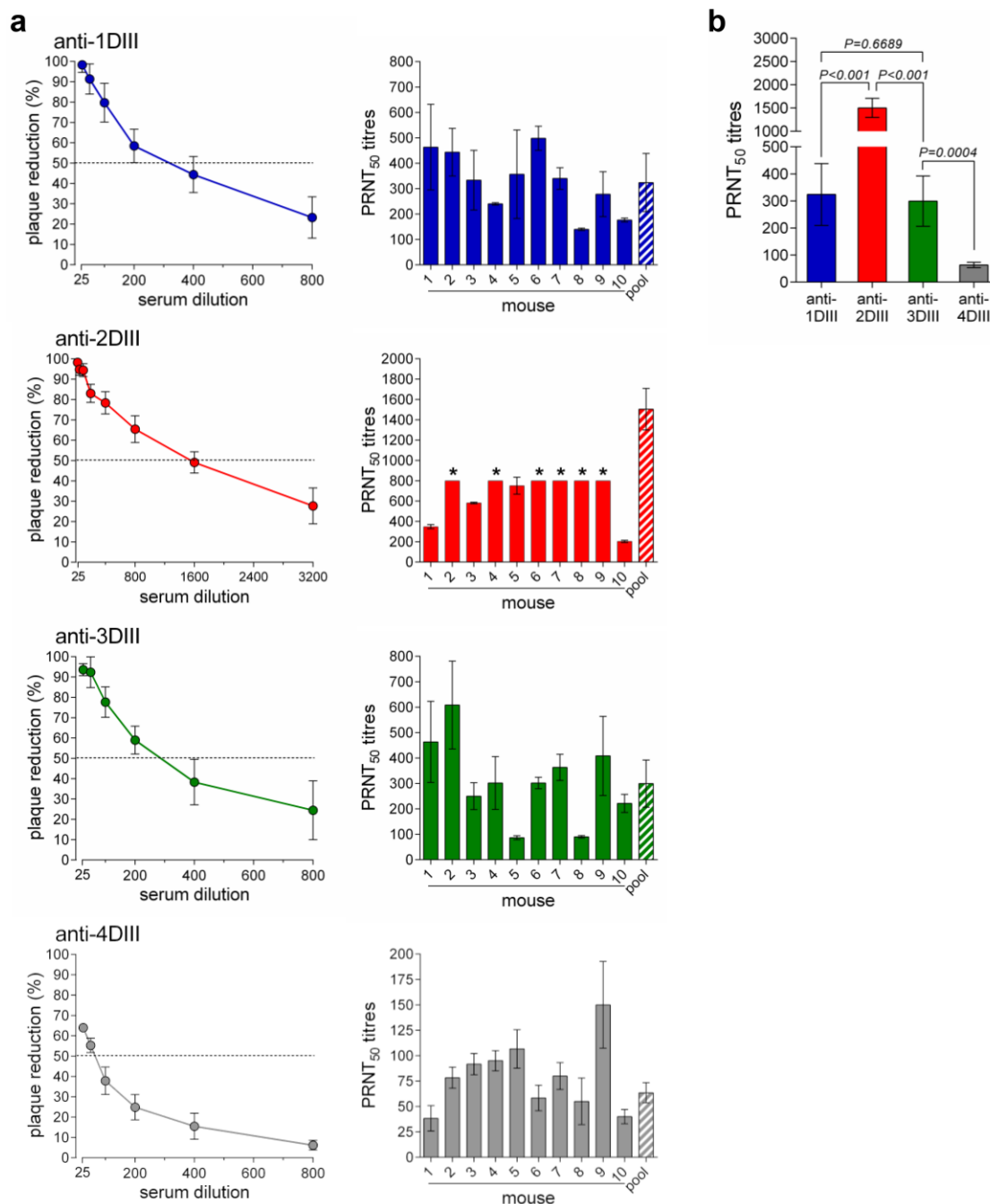


Figure 52. Neutralising capacity of anti-DIII sera. **a)** Plaque reduction curves (left panels) using anti-1DIII (n=5), anti-2DIII (n=18), anti-3DIII (n=9) and anti-4DIII (n=4) pooled sera, relative to the activity of pre-immune control sera. PRNT₅₀ titres obtained for each vaccinated animal (in all cases, n=3) are also shown (right panels) (* indicates PRNT₅₀ titre is higher than 800). **b)** PRNT₅₀ titres from curves shown in (a), comparisons between anti-1DIII and anti-2DIII (t=12,25 df=21), anti-2DIII and anti-3DIII (t=16,71 df=25), anti-1DIII and anti-3DIII (t=0,4383 df=12) and between anti-3DIII and anti-4DIII (t=4,950 df=11), are shown. In all cases, data is represented as mean±s.d. (Adapted from reference 470).

Since the neutralisation titres against DENV4 were the weakest, we assessed the possibility that factors associated with the DIII sequence used for the 4DIII- γ immunogen could have led to significant differences in the antibody response. For this, we designed a 4DIII- γ variant encoding a different DENV4 DIII sequence (DENV4 strain TC25 from

genotype I, as opposed to the Dominica strain from genotype II) with three aminoacid changes: F357L, Y360N and D384N. As shown in Fig. 53, the neutralising antibody responses induced by both constructs did not show significant differences, which suggests that strain and genotype factors are not linked, at least in this case, to the relatively reduced neutralisation induced by the 4DIII- γ vaccine.

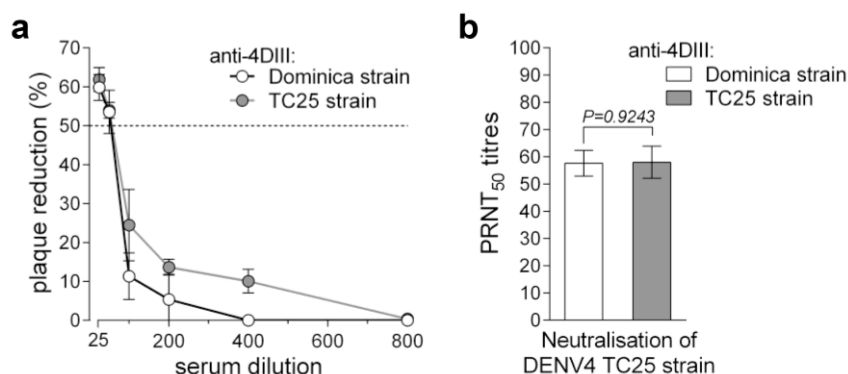


Figure 53. Effect of DENV4 strain and genotype on viral neutralisation. **a)** Plaque reduction curves on DENV4 TC25 strain using pools of sera from animals vaccinated with 4DIII- γ constructs derived from DENV4 Dominica strain (open symbols) or TC25 strain (filled symbols). **b)** PRNT₅₀ titres from curves shown in **(a)** ($n=4$; $t=0.09914$ $df=6$). In all cases, data is represented as mean \pm s.d. (Adapted from reference 470).

2.2.1.4. Cross-neutralisation and ADE activities of DIII- γ antigens.

Given that anti-DIII sera, besides anti-3DIII, showed limited cross-reactivity to DIII and sE proteins from other serotypes, we decided to also evaluate their ability to neutralise heterotypic DENV viruses. For this, all pooled sera were tested against each DENV serotype using the PRNT₅₀ in infected Vero cells. Besides confirming the previous neutralisation titres, the data shown in Table 3 revealed that, similarly to the cross-reactivity behaviour determined by ELISA, antibodies induced with the 1DIII- γ , 2DIII- γ and 4DIII- γ were not able to significantly neutralise any DENV serotype besides the homologous serotype that the antibody was raised against. In contrast, anti-3DIII antibodies showed limited cross-neutralising activity against DENV2 and DENV4 but not against DENV1.

In collaboration with Prof. Eng Eong Ooi, from the Emerging Infectious Diseases Programme at the Duke-NUS Medical School in Singapore, we performed an ADE assay with the anti-DIII sera to analyse their ability to enhance heterologous DENV infections and further describe the activities of the DIII- γ vaccines. The experiment was performed in the THP-1 human monocytic cell line expressing both Fc γ RI and Fc γ RII²⁹⁶. The results presented in Fig. 54 indicate that, for each anti-DIII serum, significant enhancement of DENV infectivity was observed only against the homologous serotype, as expected, with only limited effect on the heterologous serotypes; which suggest a high functional specificity of the antibodies induced with the DIII- γ vaccines.

Table 3. Anti-DIII cross-neutralising activity. PRNT₅₀ titres of each serotype-specific anti-DIII pooled sera on all four DENV serotypes.

Serum	Viral serotype			
	DENV1	DENV2	DENV3	DENV4
anti-1DIII	≈300	<25	<25	<25
anti-2DIII	<10	≈1600	<10	<10
anti-3DIII	<25	≈135	≈300	≈30
anti-4DIII	<10	<10	<10	≈65

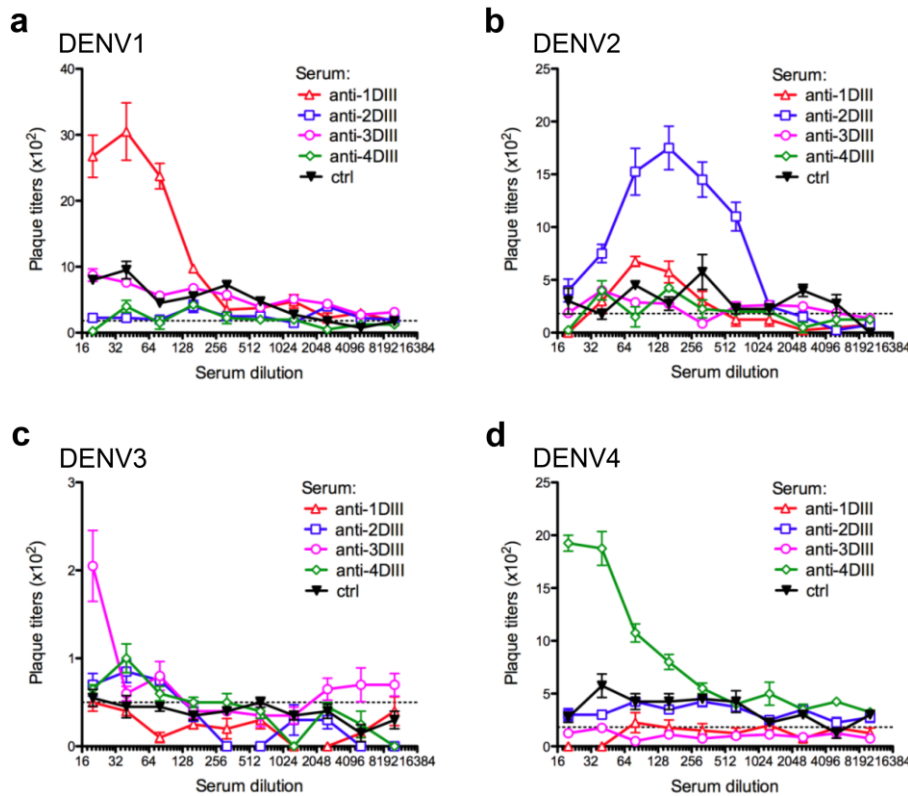


Figure 54. Comparison of ADE responses of anti-DIII sera in THP-1 monocytes. (a-d) Each anti-DIII pooled sera and a pre-immune control serum were two-fold diluted and incubated with (a) DENV1, (b) DENV2, (c) DENV3 and (d) DENV4 before infecting THP-1 cells for 72h. The culture supernatant was quantified for DENV using plaque assay afterwards. Dashed line indicates DENV baseline infection of THP-1 cells in absence of serum. Data is represented as mean±s.d (In all cases, n=3). (Adapted from reference 470).

2.2.1.5. Immune responses against DIII depend on DIII-γ secretion.

To support our findings regarding the effect of efficient antigen secretion on the immune response induced by DNA vaccines, we vaccinated mice with the non-optimised (and, as a consequence, poorly secreted (Fig. 47b)) DIII-γ constructs of DENV2, DENV3 and DENV4 (DIII^{NOp}-γ). As shown in Fig. 55, even though the encoded antigens were the same at the aminoacid level, the antibody titres obtained with the codon-optimised plasmids were considerably higher in all cases. This indicates that the efficiency showed by the DIII-γ DNA vaccines was mostly due to enhanced level of DIII secretion, and further

highlights the importance of proper antigen design before undergoing immunisation experiments, in the context of DNA vaccines.

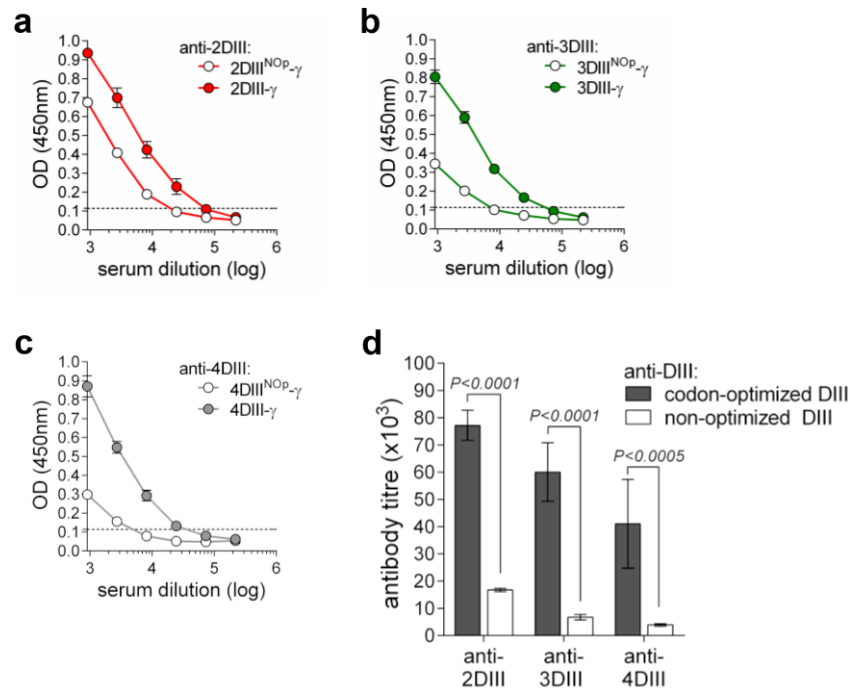


Figure 55. Increased DIII-γ secretion after codon-optimisation improves the immune responses elicited by DNA vaccination. (a-c) ELISA reactivity on the homologous DIII antigens of sera from animals gene-gun immunised with DIII-γ constructs from DENV2 (a), DENV3 (b) and DENV4 (c), with viral (DIII^{NOP-γ}) or codon-optimised (DIII-γ) nucleotidic sequences. In all cases, n=6. d) Plot of the titres from the curves shown in (a) (t=26,68 df=10), (b) (t=12,06 df=10) and (c) (t=5,585 df=10). Data is represented as mean±s.d. (Adapted from reference 470).

We then set out to determine how the design of the DIII-γ vaccine influenced the immune response elicited after DNA immunisation. For this, we compared the anti-3DIII responses induced by different constructs. As a reference, we used the preferred 3DIII-γ construct and included the non-optimised 3DIII-γ and 3DIII (3DIII^{NOP-γ} and 3DIII^{NOP}, respectively), and the 3sE construct, which encodes 3DIII along with 3DI/DII.

As seen in Fig. 56a, although all the constructs were secreted from transfected HEK-293T cells, the 3DIII-γ protein showed the most efficient secretory phenotype, while the 3DIII^{NOP} antigen was scarcely detected. Groups of 5 Balb/c mice were vaccinated following the same protocol as before, and pooled sera from each group were then evaluated in ELISA to determine anti-DIII, anti-sE and anti-DI/DII antibodies titres (to discriminate between the different specificities and allow representative comparisons, these analyses were done in plates coated with normalized amounts of biotinylated 3DIII, 3sE and 3DI/DII, respectively). As showed in Fig. 56b-c, the anti-DIII (Fig. 56b, top panel) and anti-3sE (Fig. 56b, middle panel) responses were significantly higher for the group vaccinated with 3DIII-γ, while the immune responses after vaccination with 3DIII^{NOP}, which is also the

least secreted protein, were the lowest. Anti-DI/DII antibodies were detected only in the 3sE-vaccinated animals, emphasising the specificity of anti-3DIII antibodies (Fig. 56b, bottom panel). Fig. 56d provides further details on the immune response using 3sE, which was mainly directed against DI/DII rather than DIII, confirming the immunodominance of DI/DII epitopes²⁷¹. Notably, antibodies elicited with the 3DIII- γ vaccine showed more neutralising capacity than those induced with the other constructs (Fig. 56e). This is particularly important for sE-based vaccines, since this test also measured the contribution of the anti-3DI/DII response, confirming the potential of DIII as a target for strong neutralising responses. Surprisingly, the anti-3DIII antibodies induced with different constructs differed also in their relative avidities. Sera obtained using constructs with the γ CH3 domain showed significantly higher avidity indexes than those without, suggesting that γ CH3 plays a role also in enhancing the quality of the induced antibodies (Fig. 56f).

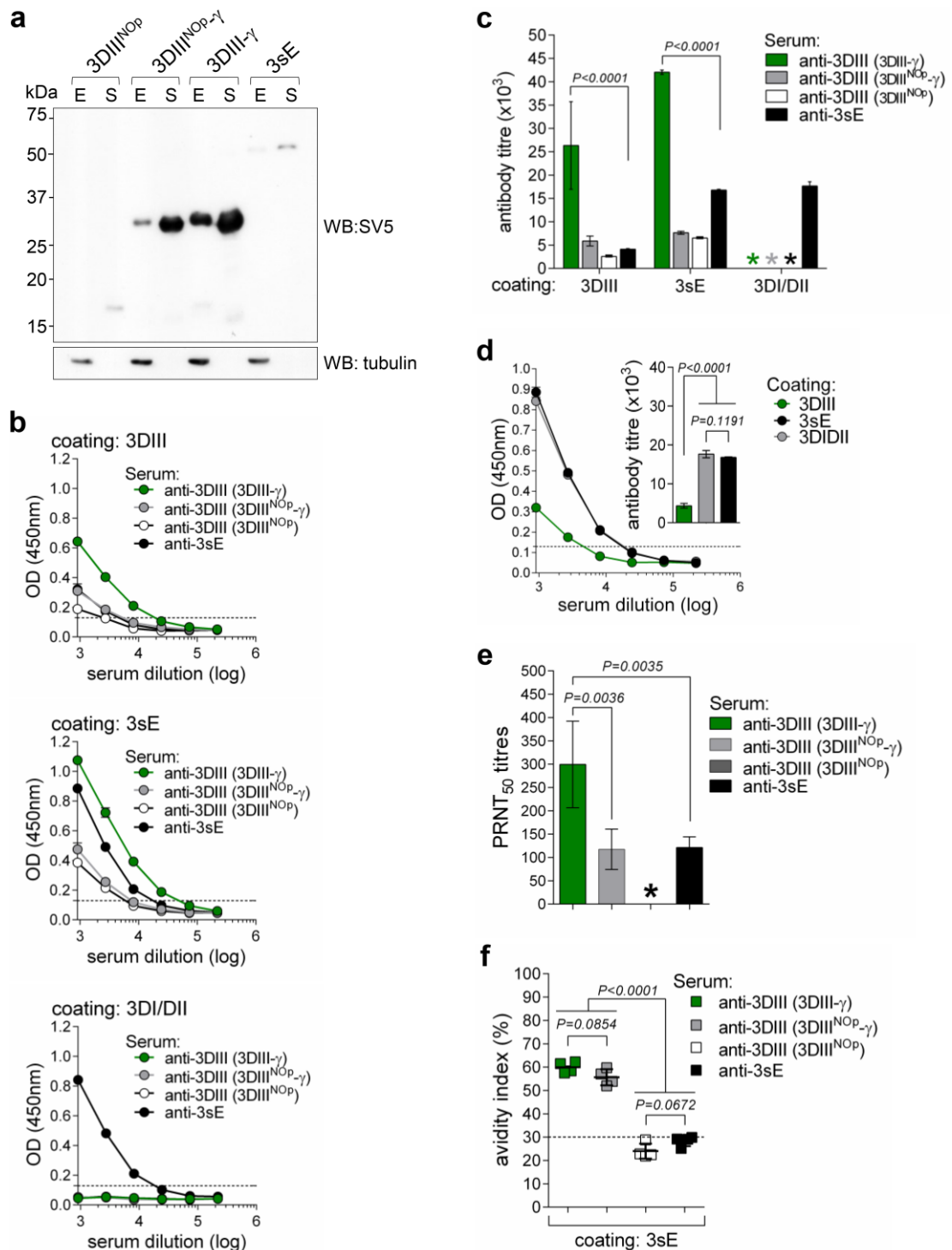


Figure 56. Effect of DIII- γ antigen design on anti-DIII antibody responses compared to 3sE. **a)** WB of total cellular extracts (E) and supernatants (S) of HEK-293T cells transfected with plasmid constructs encoding 3DIII^{NOP} (~16 kDa), 3DIII^{NOP- γ} (~28 kDa), 3DIII- γ (~28 kDa) and 3sE (~54 kDa), anti-tubulin was used as loading control. **b)** ELISA reactivity of anti-3DIII (from mice immunised with 3DIII^{NOP}, 3DIII^{NOP- γ} and 3DIII- γ) or anti-3sE on plates coated 3DIII- ϵ CH4 (3DIII), 3sE and 3DI/DII (immunising antigens indicated in parenthesis, n=4 in all cases). **c)** Antibody titres determined on each of the different coating proteins, from the curves shown in **(b)** (* indicates no reactivity at 1:300 dilution). Comparisons between anti-3DIII (3DIII- γ) and anti-3sE titres on 3DIII (t=15,51 df=6) and 3sE (t=103,0 df=6) antigens are shown. **c)** Anti-3sE sera reactivity on 3DIII, 3sE and 3DI/DII proteins as showed in **(b)**. Insert: anti-3sE titres for each coating protein. Differences of reactivity towards 3DIII and 3DI/DII (t=28,75 df=6), and against 3DI/DII and 3sE (t=1,817 df=6), are

highlighted. **e)** PRNT₅₀ titres of anti-3sE (n=4) and the different anti-DIII sera (from mice immunised with 3DIII^{NOp} (n=4), 3DIII^{NOp}- γ (n=4) and 3DIII- γ (n=9)) on DENV3. Responses from mice immunised with 3DIII- γ were compared against anti-3DIII(3DIII^{NOp}- γ) (t=3,677 df=11) and anti-3sE (t=3,704 df=11) sera. **f)** Avidity index of antibodies derived from animals gene-gun immunised with 3DIII^{NOp}, 3DIII^{NOp}- γ , 3DIII- γ or 3sE (immunising antigens indicated in parenthesis) tested on 3sE-coated plates. In all cases n=4; comparisons between 3DIII^{NOp}- γ and 3DIII- γ constructs (t=2.057 df=6), 3DIII^{NOp} and 3sE (t=2.231 df=6), and between 3DIII- γ and 3sE (t=19.76 df=6) are shown. Data is represented as mean \pm s.d. (Adapted from reference 470).

2.2.1.6. A tetravalent formulation of the DIII- γ DNA vaccine.

Since viable vaccine candidates against DENV are required to demonstrate efficient and balanced response against all four serotypes due to the risk of ADE, we next combined the four DIII- γ constructs into a tetravalent formulation that was then tested in mice using the same vaccination protocol. In this case, even though each dose of DNA was doubled (two 1 μ g shots containing an equivalent mix of 1DIII- γ and 2DIII- γ , and mix of 3DIII- γ and 4DIII- γ , respectively), the relative amount of serotype-specific DNA was halved (0,5 μ g) when compared to the monovalent responses (1 μ g). The pooled sera from vaccinated animals was then tested by ELISA and PRNT₅₀, and compared against their monovalent counterparts. As seen in Fig. 55, the tetravalent vaccine induced DIII-specific antibodies against all serotypes (Fig. 57a, left panels), albeit in significantly lower titres than those measured for the monovalent formulations. This was true for all serotypes except DENV1 where anti-DIII titres were not significantly different between both formulations (Fig. 57b). Although the PRNT₅₀ titres of the tetravalent vaccine were also reduced, the differences between the monovalent and tetravalent PRNT₅₀ titres, with the exception of DENV2, were much smaller than the ones found for the antibody titres (Fig. 57a, right panels). In addition, the avidity indexes of the antibodies induced with both formulations were comparable (Fig. 57c).

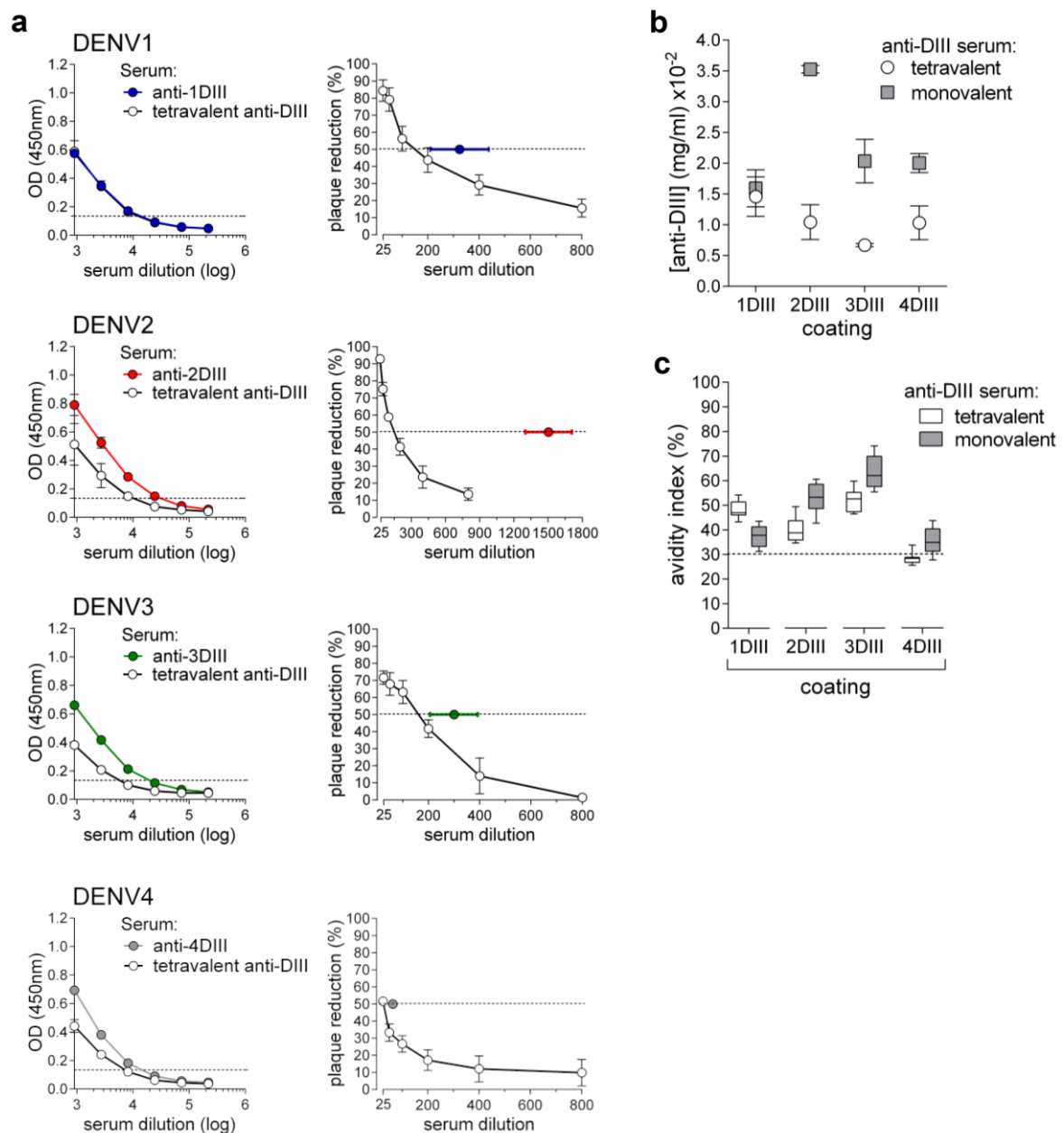


Figure 57. Tetraivalent DIII- γ formulation. **a)** ELISA (left panels, $n=4$ in all cases) and plaque reduction curves (right panels, $n=4$ in all cases) of tetraivalent (open symbols) and monovalent (filled symbols) pooled sera from gene-gun immunised mice. In right panels, curves correspond to the tetraivalent vaccine and the PRNT₅₀ titres from the monovalent immunisations (determined in Fig. 52) are shown for comparison. Data is represented as mean \pm s.d. **b)** ELISA titres (expressed as anti-DIII antibody concentrations) from tetraivalent immunisations, determined on all four DIII proteins and compared against the respective monovalent sera. Data is represented as mean \pm s.d. **c)** Avidity index of sera from monovalent ($n=13$, for all four specific sera) and tetraivalent immunisations ($n=10$ in all cases), determined on the different DIII serotype antigens. Data is represented in a box and whiskers plot. (Adapted from reference 470).

2.2.2. Optimisation of the DIII- γ DNA vaccine.

2.2.2.1. Expression of DIII- γ in pVAX1 vectors enhances antigen availability.

With the aim of further developing our DIII- γ DNA vaccine we decided to switch DNA platforms by cloning the DIII- γ constructs into pVAX1 vectors instead of the pcDNA3.1

backbone. Although pcDNA3.1 vector is commonly used it was not designed for clinical research purposes, which would prevent future evaluation of the DIII- γ candidate in an eventual Phase I study. In contrast, pVAX1 vector has a modified backbone to comply with the Food and Drug Administration (FDA) requirements for plasmid DNA vaccines against infectious diseases⁴⁷¹. Upon analysis of the secretory profile from transfected mammalian cells, production and secretion of the pVAX1-encoded constructs from transfected mammalian cells increased, when compared to profiles obtained with the pcDNA3.1 vector (Fig 58). This effect was stronger for 4DIII- γ .

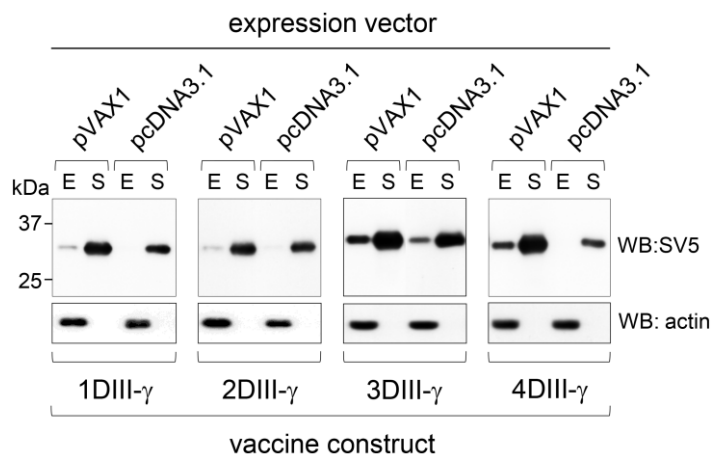


Figure 58. Cloning into pVAX1 expression vector enhances DIII- γ production and secretion from transfected cells. WB of comparable cell extracts (E) and culture supernatants (S) samples from HEK-293T cells transfected with equal amounts of the four DIII- γ constructs cloned into pcDNA3.1 or pVAX1 expression vectors. Anti-actin is included as loading control.

Given the importance of antigen secretion in DNA vaccination, and the design benefits of the pVAX1 vector, we decided to implement this format as the antigenic platform to be used for the DIII- γ DNA vaccine^e.

2.2.2.2. Evaluation of alternative vaccination protocols and DNA delivery systems.

Next we evaluated alternative DNA delivery strategies and vaccination protocols in an attempt to optimise the protection induced by the DIII- γ vaccine. For this, we measured antibody titres, avidity and neutralising capacity of sera obtained from nine groups of 6 Balb/c mice immunised with the 3DIII- γ construct in different ways and for different periods of time. The strategies, protocols and timetables used for the immunisation of each group are shown in Fig. 59.

^e From this point forward, all the experiments involving DNA gene-gun mediated immunisations were done with constructs encoded in pVAX vectors.

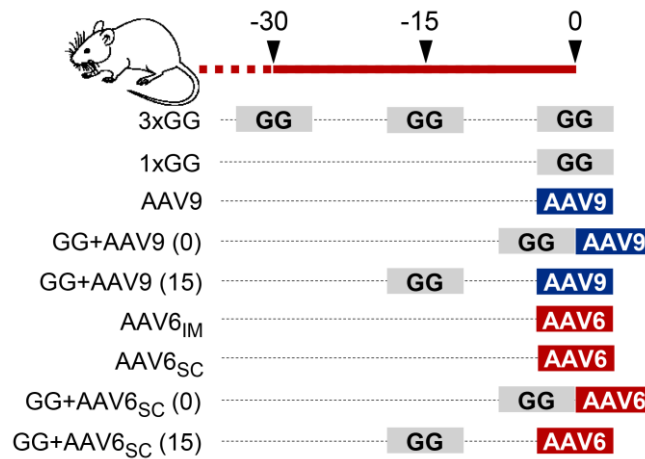


Figure 59. Schematic representation of the different vaccination strategies and protocols.

Two different serotypes of Adeno-Associated virus (AAV6 and AAV9) were introduced as an alternative DNA delivery system using single doses of 3×10^{10} viruses in both cases. In addition, AAV6 and AAV9 vectors were also implemented in combination with gene-gun (GG) delivery using prime/boost strategies where the DNA GG priming was done either previously or contemporarily to the AAV boosting (GG+AAV). Due to AAV9 preferential tropism for muscle cells⁴¹⁵, delivery was done intra-muscularly, while AAV6, that preferentially infects muscle cells but has also been described to infect keratinocytes⁴⁷², was injected both intra-muscularly and sub-cutaneously. When using the GG+AAV approach with AAV6, the sub-cutaneous route of administration was preferred to avoid similarities with the GG+AAV protocol using AAV9. We also evaluated the immunogenicity induced by a single dose of DNA as administered by GG ($1 \mu\text{g}$). The vaccination protocol used in our previous experiments (3 doses ($1 \mu\text{g}$) of biolistic-delivered DNA (3xGG)), was also included for comparison. In all cases, sera were obtained 1, 3 and 5 months after vaccination.

Antibody titres were measured on ELISA using 3DIII and 3sE as antigens (Fig.60a and 60b, respectively, upper panels). All the evaluated vaccination protocols induced anti-DIII specific antibodies, even though the immunisation efficiency was different. While the immune response in the 1xGG and AAV6_{SC} groups were the lowest, the 3xGG group showed the highest antibody titres with no significant difference between months 1 and 5. Notably, antibody titres from protocols involving AAV administration were low at month 1 with consistent increases at months 3 and 5. Among these groups, the AAV6_{IM} protocol showed the highest efficiency, even though it was still 3 times lower than titres from the 3xGG group. To determine the relative strength of the antibodies induced with each protocol, we measured sera avidity indexes on 3DIII and 3sE, 1 and 5 months after vaccination (Fig.60a and 60b, respectively, lower panels). Despite the differences in antibody titres, all protocols were able to induce high avidity responses (>30%) with higher values consistently present at month 5. For all groups, antibody titres and avidity indexes

were independent of the protein used for coating (3DIII or 3sE), further confirming that the antibodies induced by the DIII- γ antigens are able to recognise DIII in the context of the whole E protein.

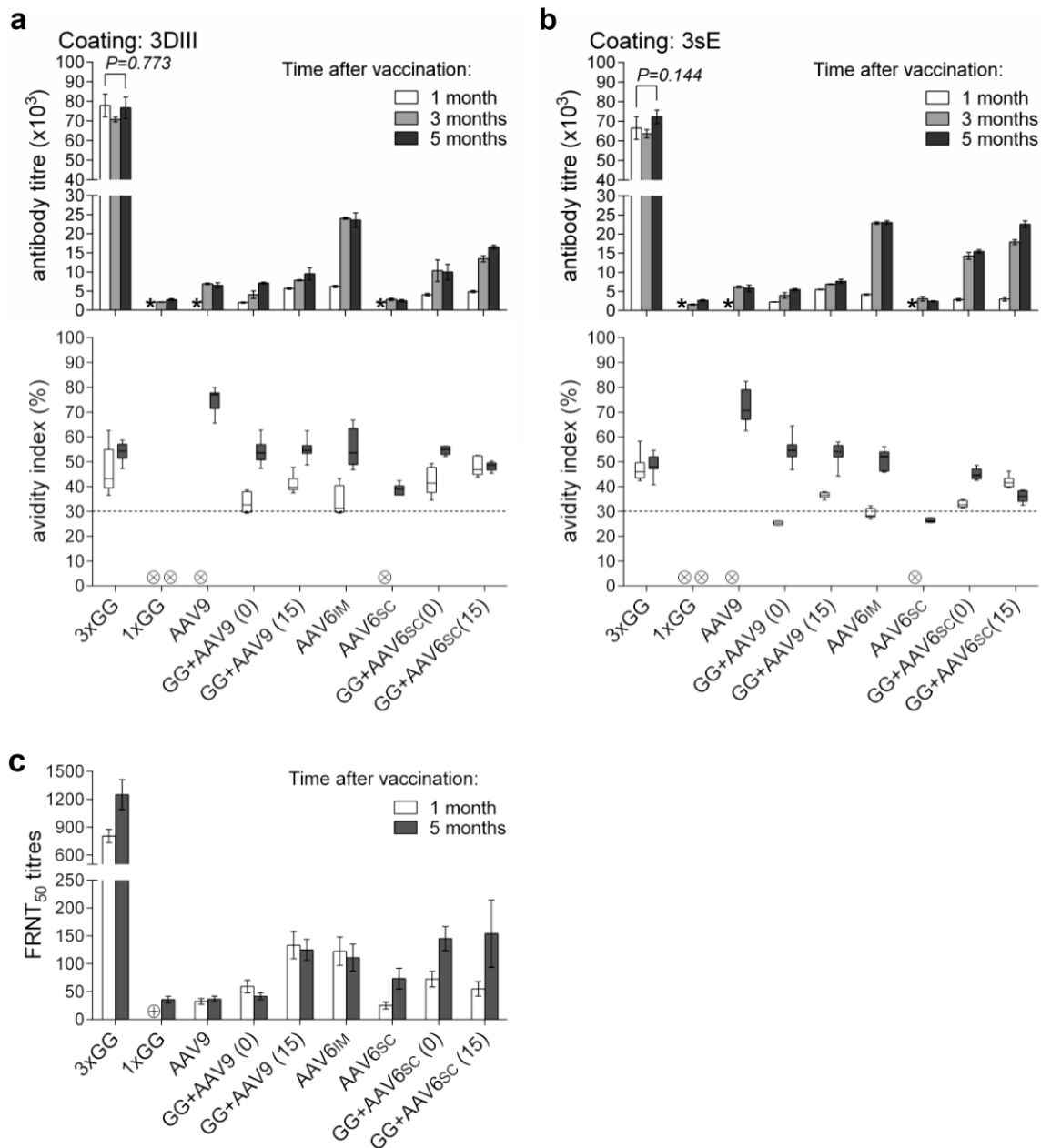


Figure 60. Characteristics of the antibody responses elicited by the different vaccination protocols. (a,b) Antibody titres measured in ELISA (top panels) and avidity indexes (bottom panels) of pooled anti-3DIII sera obtained 1, 3 and 5 months after vaccinations for the protocols evaluated, on plates coated with (a) 3DIII and (b) 3sE. In all cases, $n=4$. Antibody titres of the 3xGG protocol at months 1 and 5 were compared on 3DIII ($t=0.3017$ $df=6$) and 3sE ($t=1.680$ $df=6$). * indicates values below the 1/900 dilution threshold, (x) indicates that the avidity was not performed due to reduce reactivity in ELISA. c) Neutralisation titres of the different protocols at months 1 and 5 on DENV3 (in all cases, $n=6$, (+) indicates FRNT₅₀ below 25). Data is represented as mean \pm s.d.

We then determined neutralising activity of the antibodies produced with the different protocols using sera obtained 1 and 5 months post-vaccination (Fig. 60c). Instead of the

PRNT₅₀ assay reported previously, at this point we implemented the immunocolourimetric-based foci reduction neutralisation test (FRNT₅₀). Similar to ELISA titres, FRNT₅₀ titres of the 3xGG group were higher than all others groups. Neutralising titres above 100 were obtained for several of the groups involving AAV vectors. The neutralisation curves for each vaccination protocol are shown in Fig. 61.

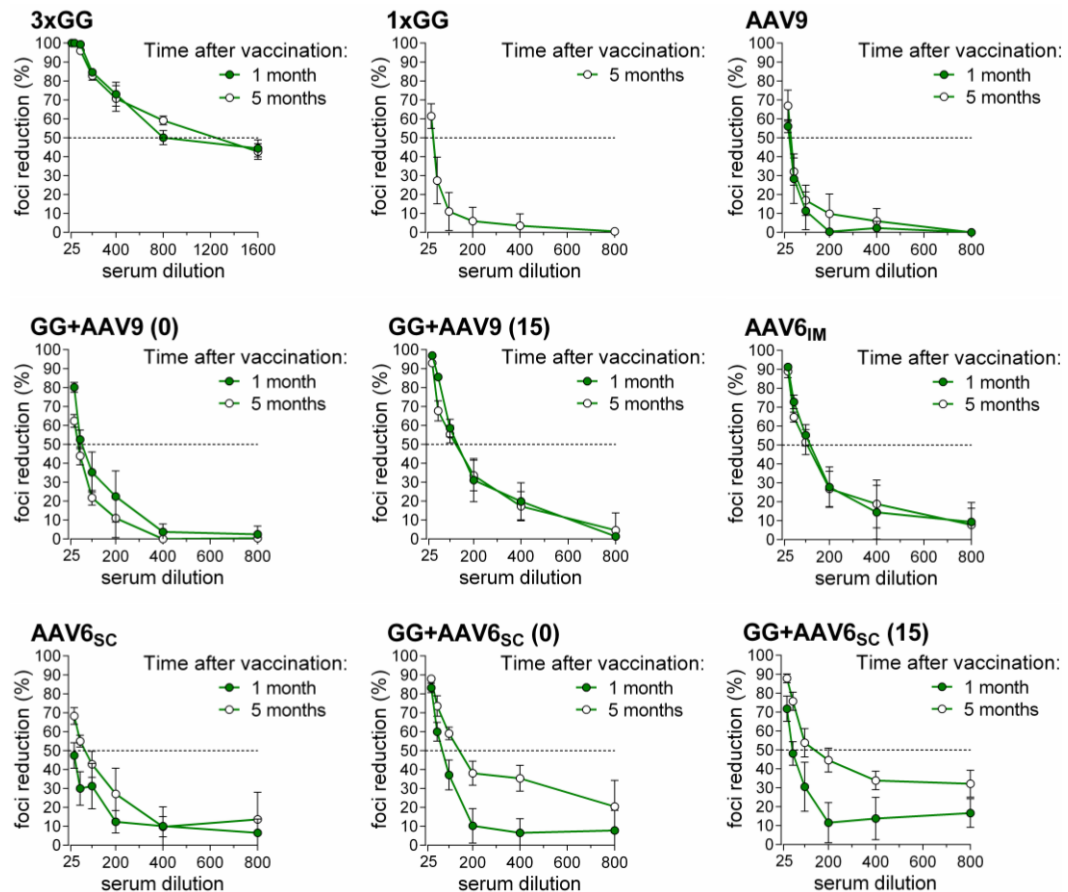


Figure 61. Foci reduction neutralisation curves for the different vaccination protocols using sera take 1 and 5 months after vaccination against DENV3 on Vero cells. In all cases, n=6 and data is represented as mean±s.d.

Even though some of the data from AAV-based protocols confirmed that AAV-mediated delivery as a valid option for genetic vaccination; these results indicate that our original 3xGG protocol was the most efficient strategy for inducing a strong neutralising antibody response with the DIII- γ DNA vaccine.

2.2.3. Longitudinal analysis of anti-DIII DNA-induced antibody responses.

2.2.3.1. Monovalent DIII- γ formulations.

After analysing the short-term immune response induced by the DIII- γ plasmids and confirming the efficiency of the 3xGG DNA vaccination protocol, we next evaluated the behaviour of the elicited antibodies over long periods of time. For this, we vaccinated

groups of 8 Balb/c mice with the DIII- γ constructs of each DENV serotype, and studied their sera for a period up to 1 year after vaccination.

Sera were obtained from each group after 1, 2, 3, 5, 8 and 12 months of completing the vaccination protocol; as seen in Fig. 62a, anti-DIII titres were determined for each group of sera, on all four DIII antigens at each time point. In agreement with our data regarding anti-DIII cross-reactivity, each group of sera showed high specificity towards the homologous DIII antigen, and only reduced cross-reactivity against the heterologous DIII. As before, the anti-3DIII response was the most cross-reactive. Notably, in all cases antibody titres against the homologous antigens showed no significant differences over the one-year period. Furthermore, the homologous anti-DIII antibody titres showed no significant differences between them when comparing the four groups at each time point (Fig. 62b). Likewise, avidity indexes determined on the homologous DIII showed little variation for all serotypes, and values remained above 30% throughout the one-year follow-up (Fig. 62c). In summary, the results indicate that gene-gun delivered DIII- γ plasmids are able to induce strong, stable and long-lasting antibody responses against each DENV serotype.

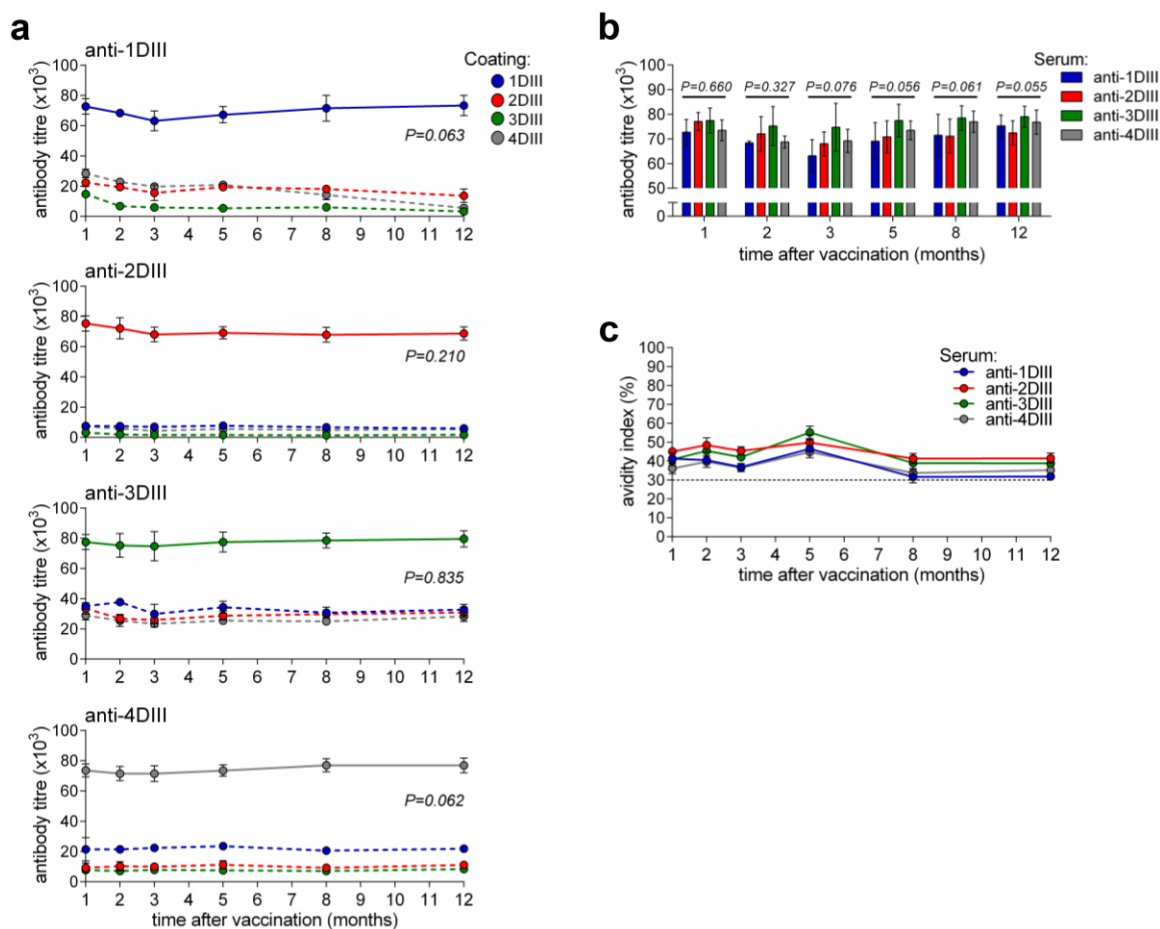


Figure 62. Longitudinal analyses of antibody responses induced with monovalent DIII- γ formulations in mice. a) Reactivity profile of serotype-specific anti-DIII sera on DIII antigens from all four serotypes during a one-year follow up (n=4 for the heterologous antigens, and n=8 for the homologous DIII in all cases). One-way

ANOVA was used to compare the antibody titres measured at each time point (anti-1DIII, $F=2.318$; anti-2DIII, $F=1.524$; anti-3DIII, $F=0.4154$ and anti-4DIII, $F=2.502$). **b)** Comparison of the antibody titres induced by the four monovalent vaccines on their homologous DIII antigens at each time point (month 1, $F=2.390$; month 2, $F=1.242$; month 3, $F=2.700$; month 5, $F=2.812$; month 8, $F=2.753$ and month 12, $F=2.846$). **c)** Time course of avidity indexes determined by ELISA, for each pool of sera, on plates coated with the homologous DIII (in all cases, $n=6$). Data is represented as mean \pm s.d.

Considering the results obtained in ELISA, we next measured the neutralising capacity of each pooled sera against their homologous DENV serotype, and evaluated their behaviour in time by comparing samples collected after 1 and 12 months of vaccination (Fig. 63a). As seen in Fig. 63b, DIII- γ constructs from all four serotypes were able to induce strong neutralising responses although, in contrast to ELISA titres, the FRNT₅₀ titres of each group were different. Importantly, the neutralising titres from all groups showed a significant increase in time, as revealed by comparing FRNT50 titres obtained at both time points. In agreement with previous results, these data indicate that antibodies induced by the DIII- γ vaccines not only maintain their neutralising capacity in time, but also show maturation-related improvement of the neutralising activity.

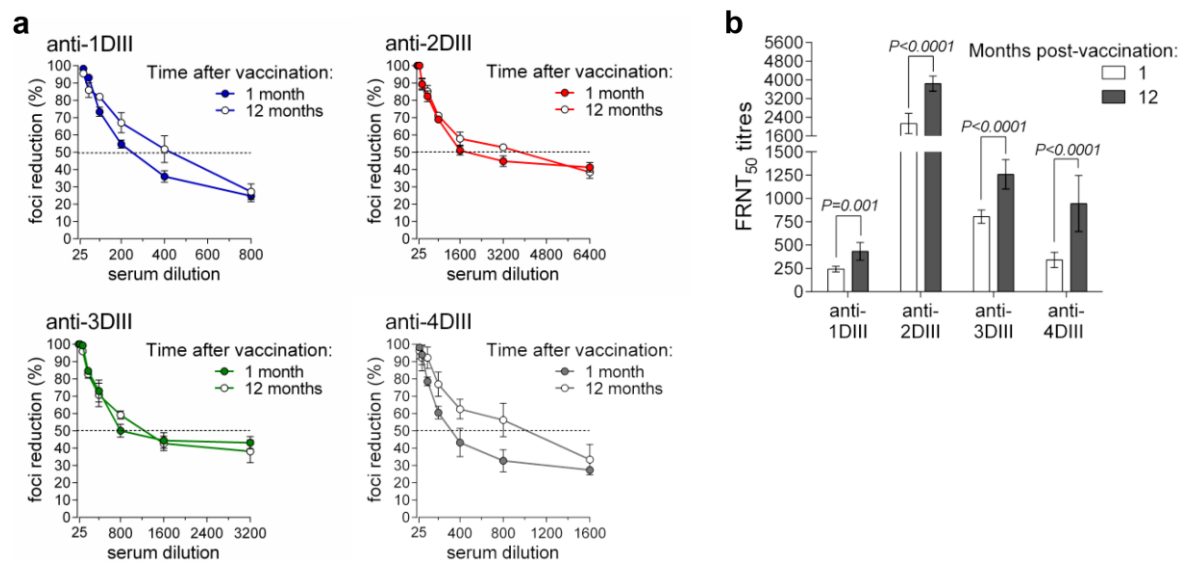


Figure 63. Longitudinal analyses of neutralising activities induced with DIII- γ monovalent vaccination in mice **a)** Foci reduction neutralisation curves for each anti-DIII against the homologous DENV serotype with sera from months 1 and 12 post-vaccination (for anti-4DIII at month 1 and 12, $n=12$ and 9, respectively; for the rest, $n=6$). **b)** Neutralisation titres of the anti-1DIII ($t=4.734$ $df=10$), anti-2DIII ($t=7.769$, $df=10$), anti-3DIII ($t=6.448$ $df=10$) and anti-4DIII ($t=6.719$, $df=19$) sera as shown in (a). Data is represented as mean \pm s.d.

2.2.3.2. Tetravalent formulations

As for the monovalent formulations and since a viable vaccine against DENV should ideally show stable efficiency against all four serotypes, we also analysed long-term antibody responses induced by tetravalent formulations of the DIII- γ constructs. Parting from our initial approach to a gene-gun delivered tetravalent DNA vaccine, we tried

different, but equivalent, formulations aimed at optimising the response obtained in our first attempt. In this case, our main objective was to develop a formulation that would efficiently deliver all four DIII-constructs and induced an immune response equivalently distributed between the four DENV serotypes. As previously mentioned, this is a critical point to consider when developing a vaccine against DENV since the elicited response should be not only protective against all four serotypes, but also balanced to avoid the risk of ADE as a result of the natural waning in antibody titres after vaccination. Since the vaccination protocol consisted of biolistic immunisation using gold particles carriers coated with DNA, we designed tetravalent formulations by either mixing plasmid DNAs, coding for different DIII- γ constructs, before coating the gold beads (DNA mix), or by mixing gold beads that had been previously coated with a single DIII antigen (Beads mix).

5 groups of 5 Balb/c mice were vaccinated following the 3xGG protocol. As before, each immunisation consisted of twice the amount of total DNA used for monovalent immunisations (two 1 μ g DNA shots), but only half the amount of each DIII construct (0,5 μ g). Group A received a DNA-mix formulation consisting of one shot with plasmids of serotypes 1 and 2, and one shot with a mix of serotypes 3 and 4 (DNA mix(1+2)(3+4)). Tetravalent formulations for groups B and C were designed using the remaining paired arrangements of the four DIII constructs; i.e. group B consisted on a DNA mix of serotypes 1+3 and serotypes 2+4 (DNA mix(1+3)(2+4)), while group C involved a DNA mix of serotypes 1+4 and 2+3 (DNA mix(1+4)(2+3)). Group D received a formulation consisting of two equal shots of gold beads coated with a mix of all four DNAs (DNA mix(1+2+3+4)). Instead, mice from group E received a beads-mix formulation consisting of one shot with a mix of gold particles coated with plasmids for 1DIII- γ and 2DIII- γ , and one shot of a mix of particles coated with plasmids for 3DIII- γ and 4DIII- γ (Beads mix(1+2)(3+4)).

Animals were bled at different time points and antibody titres against each DIII antigen were determined (Fig. 64a-e). For all groups, immune responses to all constructs were essentially maintained throughout the one-year follow up. However, specific DIII titres were lower in all formulations when compared to monovalent responses. This is consistent with our previous results and with the reduced amount of serotype-specific DNA used during immunisations. We noticed some similarities among the antibody responses obtained in all groups: while anti-3DIII titres were consistently the lowest, responses towards 1DIII and 2DIII were the highest. Responses at month 1 for all formulations were unbalanced and normalised in time. Notably, mice from group E (beads mix) developed an antibody response that was equally distributed among the four DIII serotypes, while anti-3DIII responses were consistently lower in all groups involving DNA-mix formulations. Together, the results indicate that vaccine formulation has an important effect on the immune responses.

Using sera from groups A and E, which involved the same pairing of serotype-specific plasmids but differed in the way of preparation (DNA-mix for group A, Beads-mix for group E), we measured the neutralisation titres against all DENV serotypes and evaluated their variation in time by comparing samples obtained at month 1 and 12 after vaccination. As seen in Fig. 64f, relevant neutralising activity was detected against all serotypes in both groups. Compared to monovalent responses, neutralising activity of both groups towards DENV2, DENV3 and DENV4 were lower, while neutralisation of serotype 1 was enhanced but only in mice vaccinated with the Beads-mix formulation. Of note, while the neutralisation of serotypes 2, 3 and 4 were similar in both groups, the response against DENV1 was a significantly higher for the beads-mix formulation. Tetravalent neutralising responses were not significantly different at the beginning and end of the one-year study, highlighting the stability of the immune responses induced with the tetravalent formulations.

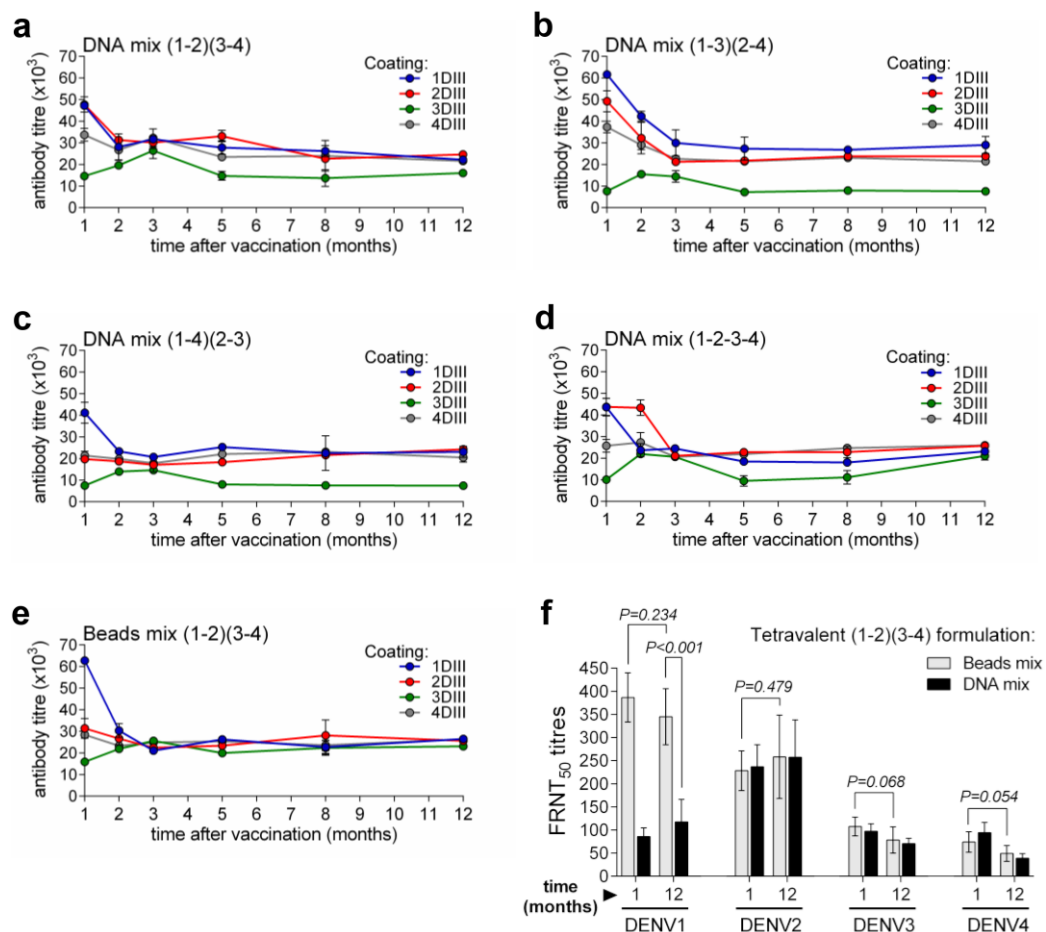


Figure 64. Longitudinal analysis of antibody responses induced with different tetravalent formulations in mice. (a-d) Antibody titres of the different tetravalent formulations as determined in ELISA against the four DIII antigens with pooled sera samples at each time point ($n=8$ in all cases). **f)** Neutralisation titres of sera from animals vaccinated with DNA mix (1-2)(3-4) (corresponding to (a)) and Beads mix (1-2)(3-4) (corresponding to (e)) tetravalent formulations against the four DENV serotypes using samples taken 1 and 12 months after vaccination (in all cases, $n=6$). Comparison of both formulations against DENV1 is shown ($t=13.07$ $df=10$); for the beads mix group the neutralising responses showed no significant differences in time

($t=1.267$ $df=10$ for DENV1, $t=0.7358$ $df=10$ for DENV2, $t=2.050$ $df=10$ for DENV3 and $t=2.181$ $df=10$ for DENV4). Data is represented as mean \pm s.d.

2.2.4. Functional dissection of the antibody response against DENV sE.

2.2.4.1. DI/DII epitopes dominate the immune response against sE.

The data so far presented confirmed that DNA immunisation with DIII- γ constructs induce long-lasting, strongly neutralising antibodies against the homologous DENV serotype with only limited cross-reactivity and ADE capacity against heterotypic serotypes. In addition, we have also showed that, in agreement with other studies^{271,278}, anti-sE responses consist mainly of antibodies with anti-DI/DII activity and very limited reactivity to DIII.

To complete the study of the immune responses induced by the different E structural domains, and assess their potential use as immunogens in DNA vaccines, we decided to vaccinate animals with constructs encoding the DIII- γ antigen, DI/DII or the complete sE protein of DENV3 and DENV4 (Fig. 65a). DENV1 and DENV2 were not included as only DI/DII and the sE proteins from serotypes 3 and 4 were efficiently secreted from mammalian cells (Fig. 22a). The secretory properties of these constructs in the pVAX1 vector were confirmed after transfection in HEK-293T (Fig. 65b).

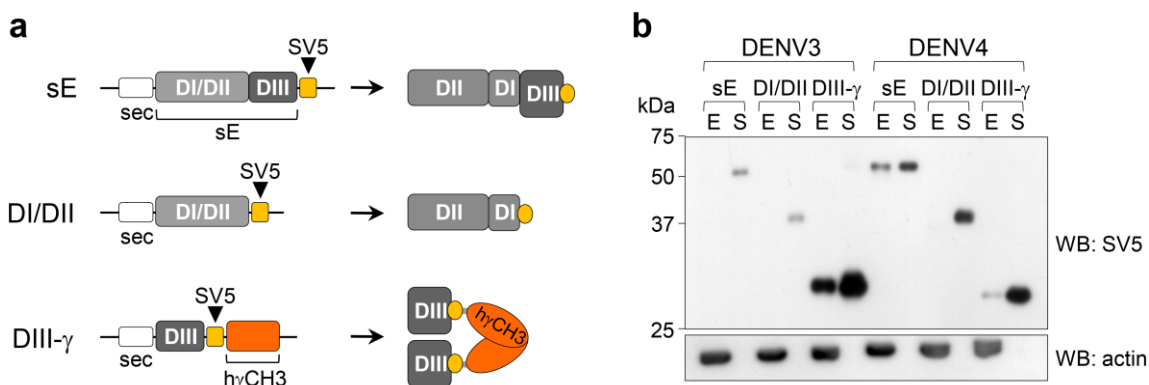


Figure 65. Secretory profiles of sE, DI/DII and DIII- γ proteins derived from DENV3 and DENV4. **a)** Scheme of DNA constructs and the expected products used for immunisations. **b)** WB profile of expression and secretion from transfected HEK293T cells (E, cellular extracts; S, culture supernatants) of the constructs shown in **(a)**; anti-actin was used as loading control.

Pooled sera of immunised animals were then tested by ELISA on plates coated with the homotypic sE, DI/DII or DIII antigens to determine the reactivity profile of the induced antibodies. As seen in Fig. 66a, for both DENV3 and DENV4, anti-DIII and anti-DI/DII sera were highly domain-specific, while both anti-sE sera preferentially reacted with DI/DII and sE, as expected due to DI/DII immunodominance. Indeed, there were no significant differences between titres of anti-sE sera determined on sE and DI/DII, while the differences between titres determined on sE and DIII were (Fig. 66b and 66c).

Furthermore, both anti-DI/DII antibodies showed significantly lower avidity compared to antibodies induced with DIII- γ (Fig. 66d).

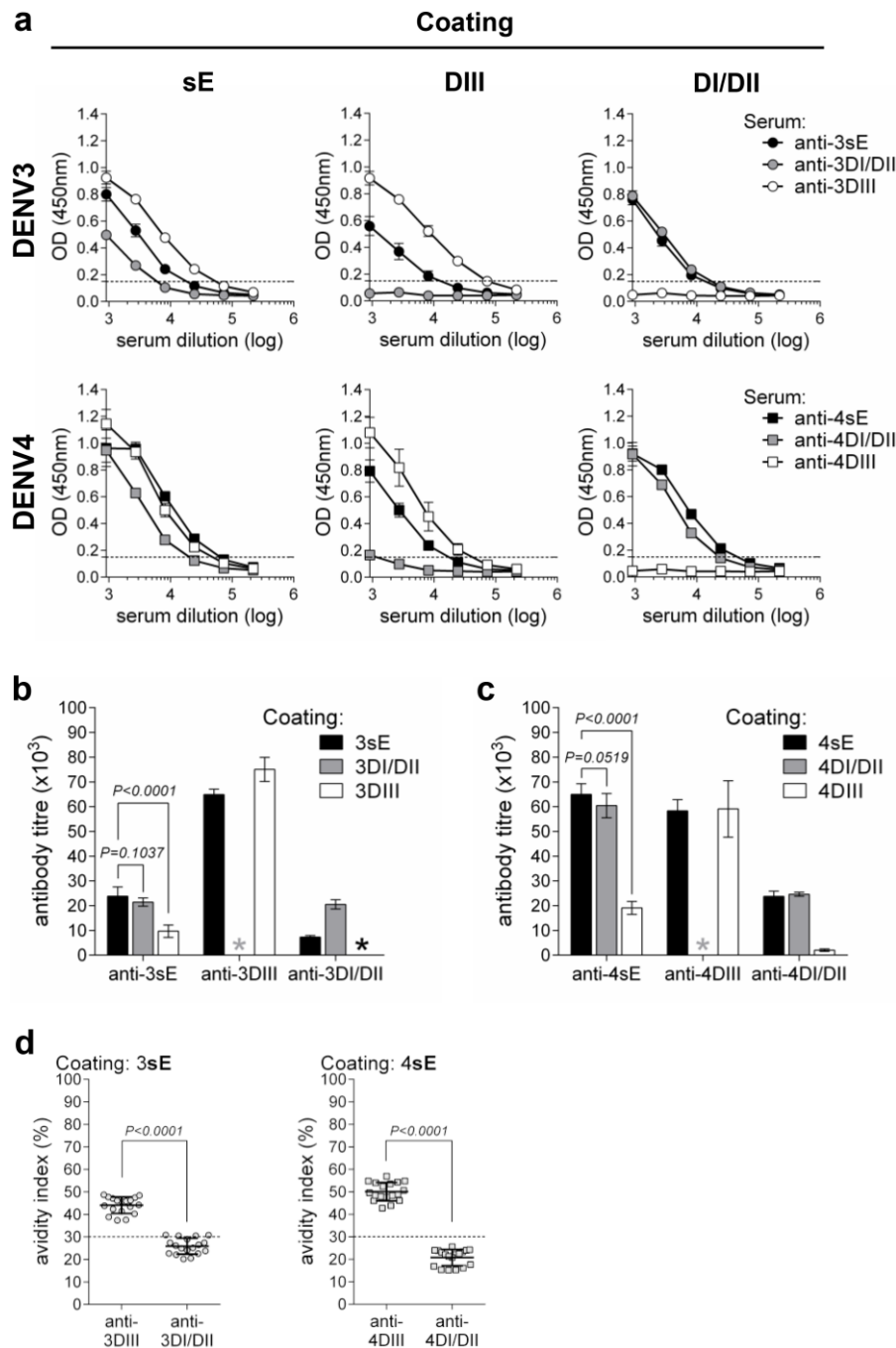


Figure 66. Antibody responses against DENV sE antigens are highly dominated by DI/DII epitopes. a) ELISA reactivity of antibody responses from mice immunised with sE (n=12), DI/DII (n=9) and DIII (n=9) constructs from DENV3 and DENV4, tested on plates coated with sE, DIII or DI/DII antigens from the homologous serotype (DENV3, top panels) and (DENV4, bottom panels) as indicated. **(b, c)** Antibody titres of sera from animals immunised with constructs of DENV3 **(b)** and DENV4 **(c)** as determined in **(a)**, comparisons of anti-sE reactivity between sE and DI/DII ($t=1.714$ $df=19$ for DENV3, $t=2.082$ $df=19$ for DENV4), and between sE and DIII ($t=6.361$ $df=19$ for DENV3, $t=28.16$ $df=19$ for DENV4) are shown. **d)** Avidity index of sera from animals immunised with DIII and DI/DII from DENV3 (left panel, $n=18$, $t=15.10$ $df=34$) and DENV4 (right panel, $n=18$, $t=22.07$ $df=34$). Data is represented as mean \pm s.d.

In addition, as demonstrated by the analysis of sera from animals vaccinated with both DIII- γ and DI/DII constructs (groups of 6 Balb/c mice, following the same vaccination protocol, with animals receiving 1 μ g DNA/shot) DI/DII immunodominance was only observed within the context of the whole sE protein (in *cis*) but not when DIII and DI/DII were expressed separately (in *trans*). As shown in Fig. 67a, addition of DI/DII had no significant effect on the antibody titres against DIII (Fig. 67a). Antibody titres against 3DI/DII are also included to demonstrate the presence of anti-DI/DII antibodies in the group vaccinated with both constructs (Fig. 67b).

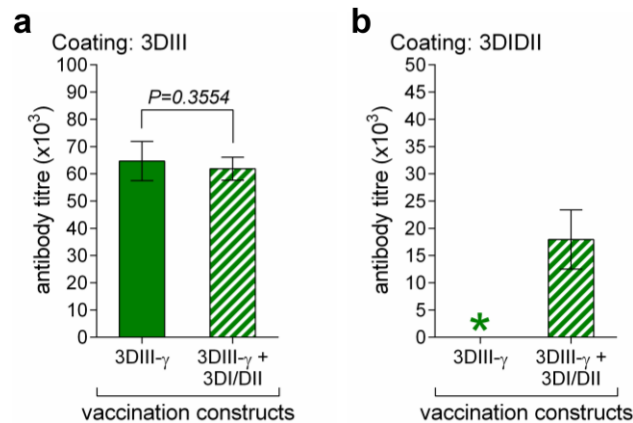


Figure 67. DI/DII immunodominance on DIII is observed only with the context of sE. a) Antibody titres on 3DIII-coated ELISA plates of pooled sera from animals vaccinated only with 3DIII- γ or with 3DIII- γ and 3DI/DII in two different shots (n=8, t=0.9558 df=14). b) ELISA reactivity of the same sera on 3DI/DII coated plates (n=8). Data is represented as mean \pm s.d.

2.2.4.2. Antibodies induced against DI/DII, but not against DIII are highly cross-reactive to other flaviviruses.

We then investigated the reactivity profile of the immune sera by immunofluorescence of cells infected with all DENV serotypes and with other three closely related flaviviruses (ZIKV, WNV and YFV). In addition to the sera of animals vaccinated with all four DIII- γ constructs, and DI/DII and sE from serotypes 3 and 4, we also included sera from mice vaccinated with the tetravalent DIII- γ beads-mix formulation (henceforth, Tetra-DIII) described before.

As stated in previous sections, anti-DIII antibodies were highly reactive when exposed to cells infected with their homologous DENV serotype; predictably, sera antibodies induced with the Tetra-DIII formulation were also able to recognise all four DENV serotypes. Likewise, anti-DI/DII and anti-sE sera, from both DENV3 and DENV4, were highly cross-reactive to cells infected with every serotype, an expected feature given the DI/DII homology of DENV viruses^{271,278} (Fig. 68). mAb 4G2 and pre-immune sera were used as positive and negative controls, respectively.

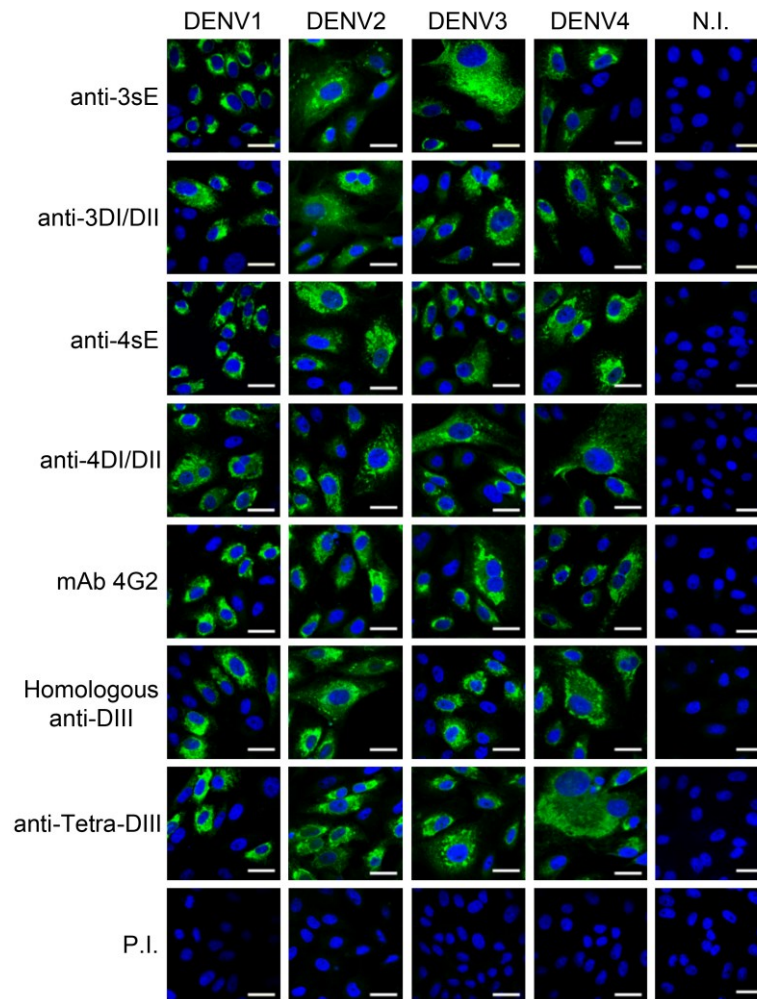


Figure 68. Reactivity profiles of different anti-DENV sera in DENV-infected cells. Immunofluorescence of Vero cells infected with all four DENV serotypes and reacted with antibodies elicited with 3sE and 4sE or the corresponding 3DI/DII and 4DI/DII. mAb 4G2 as well as the homologous DIII and tetravalent anti-DIII sera were included as controls. N.I., non-infected cells; P.I., pre-immune serum. Bar, 30 μ m.

Strikingly, when the same sera were analysed on cells infected with the non-dengue flaviviruses the reactivity profiles were different. Sera of mice immunised with DI/DII and sE constructs, from both serotypes, remained strongly cross-reactive to ZIKV, WNV and YFV. In contrast, anti-DIII antibodies obtained from groups vaccinated with each DIII- γ protein or with the Tetra-DIII formulation were completely negative to all three viruses (Fig. 69). As before, given that the FL epitope is conserved in the three viruses⁴⁴⁰, mAb 4G2 and pre-immune sera were used as a positive and negative controls, respectively.

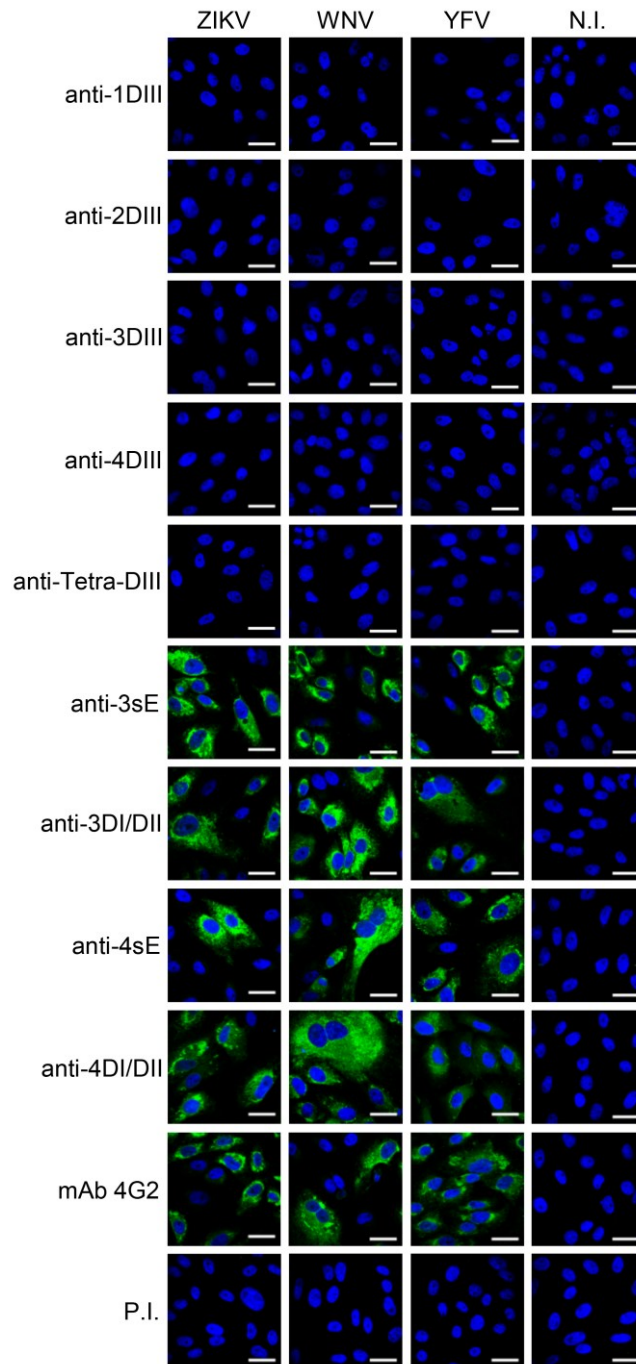


Figure 69. DENV anti-DIII sera recognise DENV infected cells specifically and do not cross-react with other flaviviruses. Immunofluorescence of Vero cells infected with ZIKV, WNV and YFV and reacted with antibodies elicited with the four DENV DIII antigens (1DIII, 2DIII, 3DIII and 4DIII), the Tetra-DIII formulation, 3sE, 3DI/DII, 4sE and 4DI/DII. mAb 4G2 was included as a control. N.I., non-infected; P.I., pre-immune serum. Bar, 30 μ m.

2.2.4.3. Neutralising and enhancing activities of polyclonal antibodies against DENV DIII and DI/DII.

Given the clear contrast between the reactivity profiles of the broadly cross-reactive antibodies induced with proteins containing DI/DII epitopes (DI/DII and sE) and the highly

specific responses against immunogens restricted to DIII, we then evaluated their performance in ADE and FRNT₅₀ assays (using K562 and Vero cells, respectively) against all the aforementioned viruses. A control of ADE assay on K562 cells was done with mAb 4G2, which showed enhancing activity to all viruses (Fig. 70).

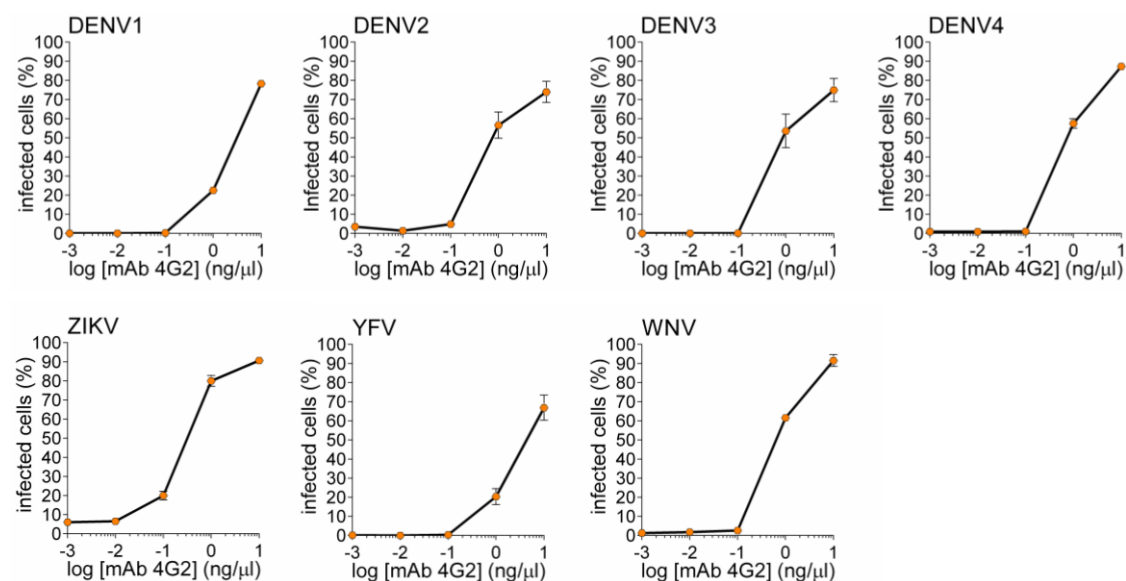


Figure 70. mAb 4G2 induces ADE of all four DENV serotypes, ZIKV, WNV and YFV on K562 cells (n=3).

In order to successfully dissect the activities of the immune responses induced with defined E structural domains, and since the reactivity profiles of anti-DI/DII and anti-sE sera (for both DENV3 and DENV4) were the same regardless of the virus, we only compared anti-DI/DII with anti-DIII specific sera.

As seen in Fig. 71a, we found strong ADE activities for both anti-DI/DII sera (anti-3DI/DII and anti-4DI/DII) not only for the different DENV serotypes, but also against ZIKV, WNV and YFV. Interestingly, the overall patterns of the neutralising activity were different, as viral neutralisation was relevant only against DENV and completely negative on the other flaviviruses. The summary of the neutralisation titres for both anti-DI/DII is shown in Fig. 71b.

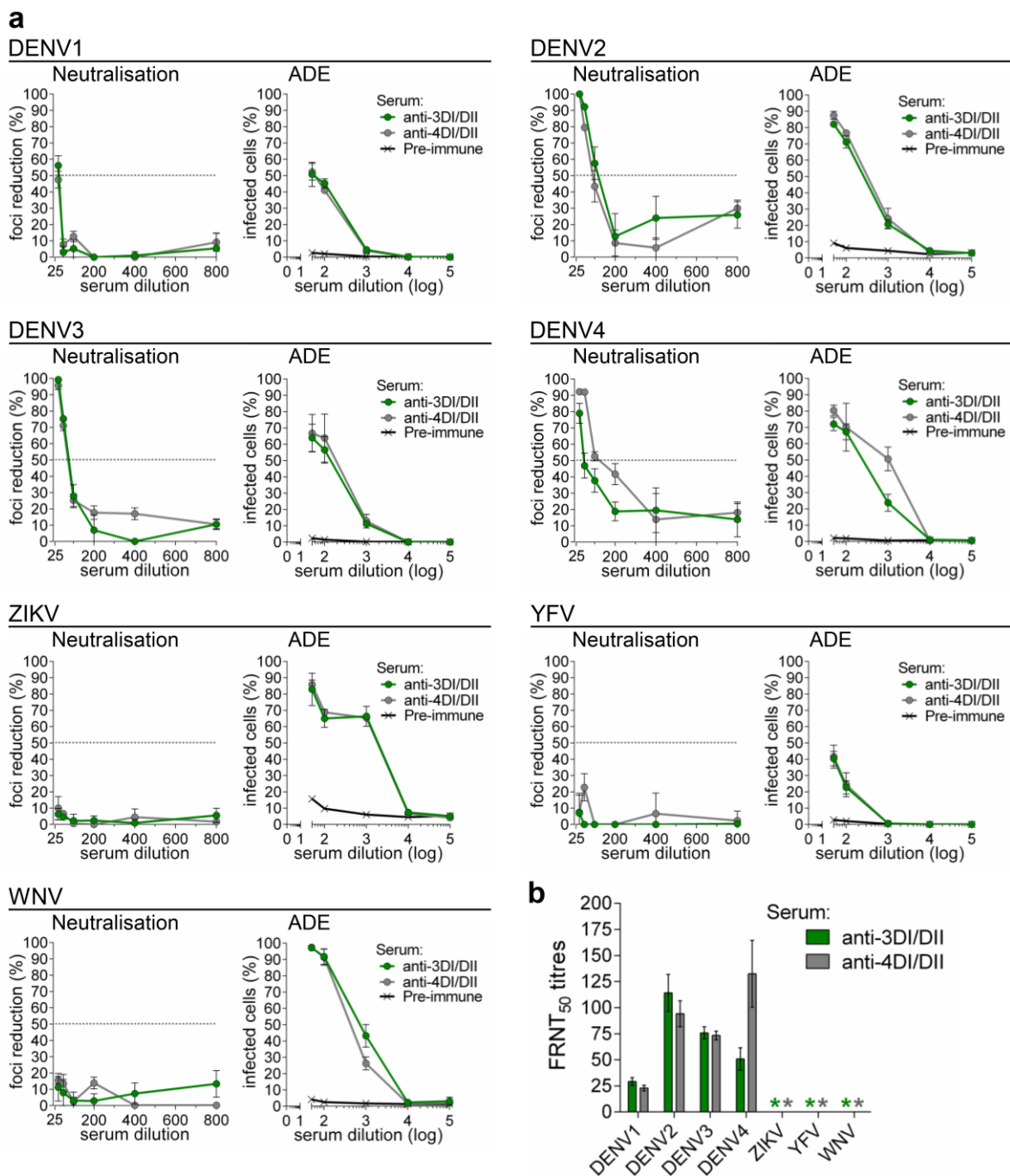


Figure 71. Cross-reactive anti-DI/DII induces poorly-neutralising and highly-enhancing responses. a) Foci reduction neutralisation curves (n=6) and ADE (n=3) of all four DENV serotypes, ZIKV, WNV and YFV, with DENV anti-3DI/DII and anti-4DI/DII sera. **b)** FRNT₅₀ titres for anti-DI/DII sera derived from the curves shown in (a), * indicates FRNT₅₀ titres below detection limit of the assay (<25).

In contrast to the profiles obtained with anti-DI/DII antibodies, immune responses induced with the different DIII- γ proteins showed no reactivity, in both ADE and FRNT₅₀, when tested against ZIKV, WNV and YFV (Fig. 72a). As expected, strong neutralisation and, consequently, ADE activity, was found when testing each anti-DIII sera against their homologous DENV serotype. The neutralisation titres of each anti-DIII sera are summarised in Fig. 72b.

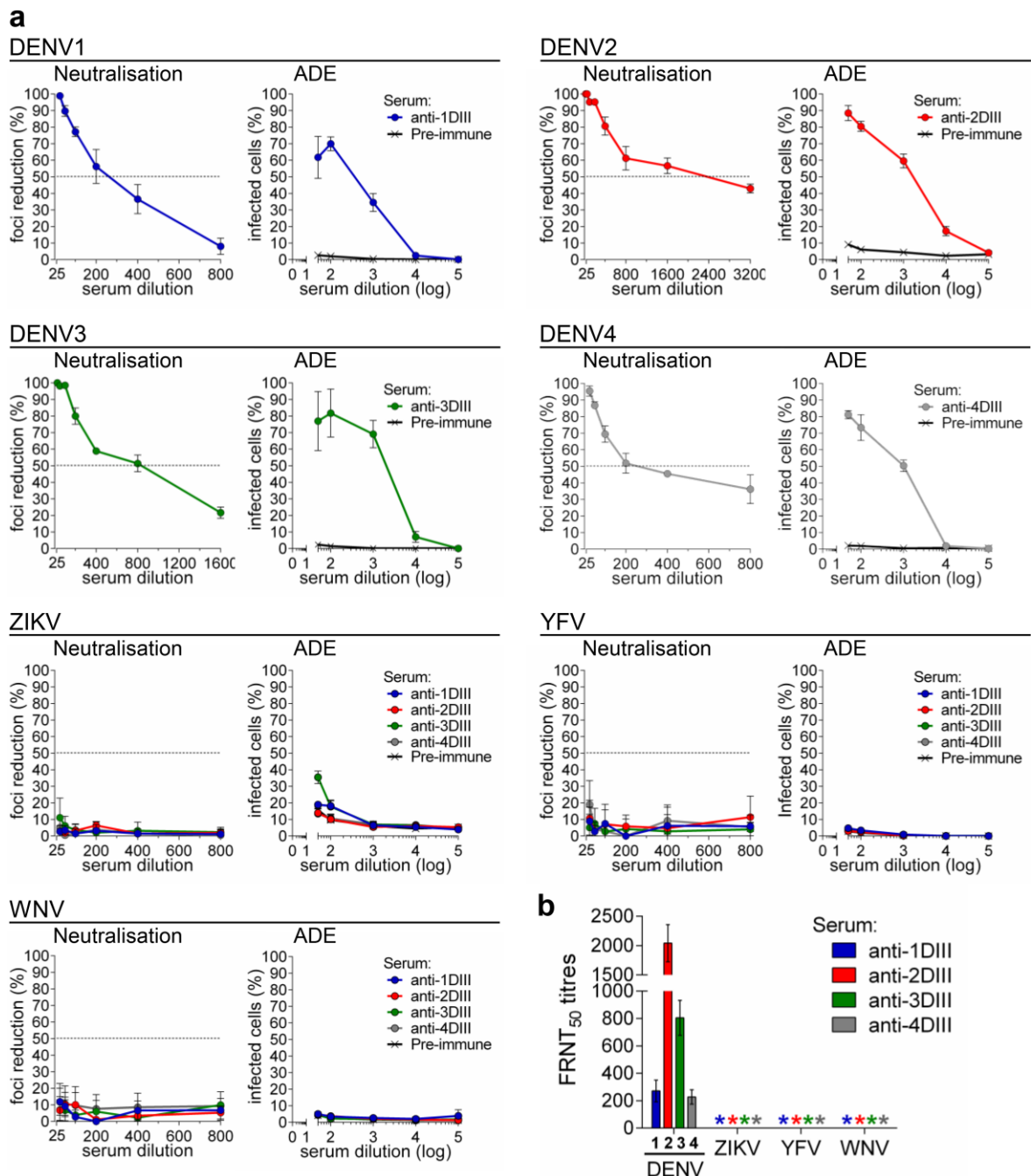


Figure 72. DIII- γ DNA vaccination induces DENV-specific neutralising antibodies. a) Foci reduction neutralisation (n=6) and ADE (n=3) of all four DENV serotypes, ZIKV, WNV and YFV, with DENV anti-DIII sera. DENV viruses were tested using sera against the homologous serotypes while non-DENV viruses were tested against all four anti-DIII sera. **b)** FRNT₅₀ titres for anti-DIII sera as shown in (a), * indicates FRNT₅₀ titres below detection limit of the assay (<25).

To further confirm the DENV specificity of the DIII- γ immunogens, we then determined the neutralising and ADE activities of antibodies induced with the Tetra-DIII formulation. Not surprisingly, anti-Tetra-DIII sera showed no neutralising activity against ZIKV, WNV and YFV, but were able to induce significant neutralisation to the four DENV serotypes. Notably, there was no ADE activity towards the non-dengue flaviviruses; while, as

expected, enhancement of DENV serotypes was positive (Fig. 73a). FRNT₅₀ titres of anti-Tetra-DIII sera are shown in Fig. 73b.

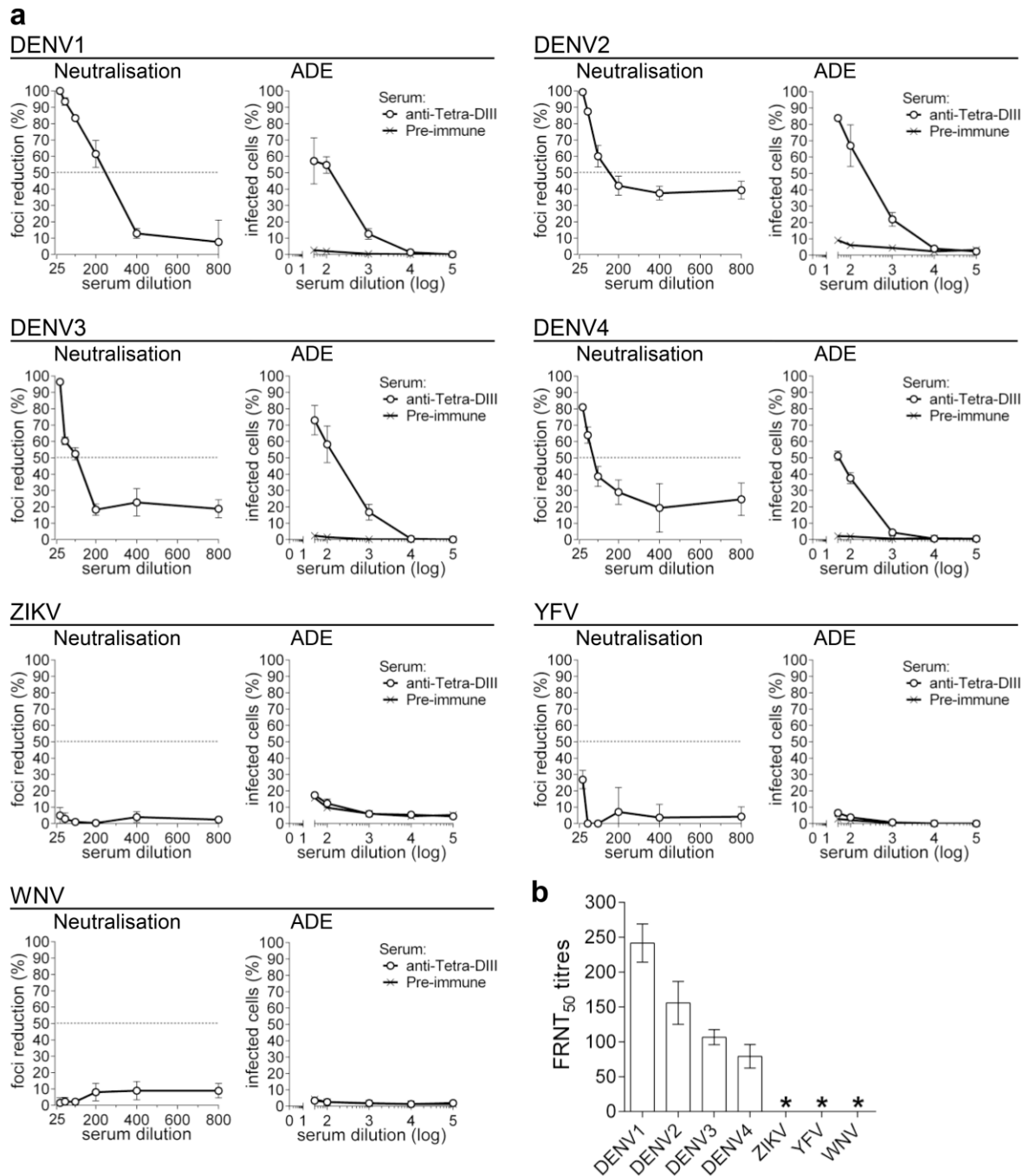


Figure 73. DENV tetraivalent DIII- γ vaccine formulation elicits DENV-specific neutralising antibodies. a) Foci reduction neutralisation curves (n=6) and ADE (n=3) of all four DENV serotypes, ZIKV, WNV and YFV, with sera from mice vaccinated with the Bead mix(1-2)(3-4) tetraivalent DIII- γ formulation. b) FRNT₅₀, as determined in (a), * indicates FRNT₅₀ titres below detection limit of the assay (≥ 25).

Together, these data support the implementation of DIII antigens to induce DENV-specific neutralising responses without the risk of enhancing infection of other related flaviviruses like ZIKV, WNV and YFV.

2.3. DISCUSSION

Even though the development of an effective Dengue vaccine has been a highly sought objective for decades, it remains an unresolved task. The lack of proper animal models and laboratory tests able to reliably extrapolate data to the clinical reality^{337,473}, together with the poor understanding of the infection process of the virus, have been major caveats to circumvent²²⁰. Furthermore, the need to develop a candidate able to elicit a balanced and stable neutralising response to all four DENV serotypes due to the risk of ADE has hindered many efforts to move more viable vaccine candidates down the pipeline.

Recently, the promising CYD-TVD vaccine from Sanofi-Pasteur has been seriously questioned due to unbalanced protection against the different DENV serotypes and increased risk for haemorrhagic disease among children between 2-5 years of age^{368,369,371}. Thus, as a result of the limitations shown by classical vaccine approaches, next-generation strategies have emerged as new alternatives to overcome this complex task with new candidates based on recombinant VLPs³⁹², molecularly attenuated live viruses^{356-358,361-363}, viral-vectored genetic vaccines and tetravalent DNA vaccines³⁴², all moving closer to Phase III studies. In fact, the current urge to quickly address the need of an effective vaccine against ZIKV has led to the development of several genetic vaccine candidates (both as DNA and viral-vectored vaccines) that are swiftly moving into clinical studies ahead of other, more traditional, candidates^{453-455,474,475}. In addition, DNA vaccines against other flaviviruses like WNV⁴⁰⁵, and against viral diseases like human immunodeficiency virus (HIV) and Ebola virus, have also been clinically tested in humans^{402,404}.

As mentioned before, genetic vaccines are able to stimulate cellular and antibody-mediated adaptive immunity, which is among their main advantages³⁹⁵⁻³⁹⁷. Other benefits of DNA vaccines, especially relevant when dealing with rapidly spreading viruses in underdeveloped countries like DENV and ZIKV, include: a) exceptional safety profile in humans, b) the possibility to screen several antigenic designs in short periods of time, c) fast manufacturing and d) regulatory simplicity for clinical evaluation^{395,398,454,475}. In addition, given that the antigen is synthesised intracellularly *in vivo*, proper conformation of the epitopes is favoured since the immunogen undergoes the same maturation process as the native viral protein would during infection³⁹⁸⁻⁴⁰⁰.

Although most DNA vaccine candidates in development include the whole E protein as the main antigen, we show that DNA vaccination with properly engineered antigens based on DIII induced strong antibody responses to all DENV serotypes in mice. The results confirm previous indications regarding the neutralising properties of anti-DIII antibodies and the reduced risk of ADE due to their specificity⁴⁷⁶. It is important to mention that due to the lack of resources and appropriate facilities to handle infected

animals, the study of the vaccine was restricted to the Balb/c immunocompetent mouse model; a more complete evaluation of vaccine effectiveness should have included protection studies in passively immunised AG129 mice.

In order to enhance anti-DIII responses, we took an integral approach in designing the DIII immunogen. Based on the biochemical analysis done on sE proteins from the different DENV serotypes (and their respective structural domains), our data clearly indicate that efficient antigen production and secretion from transfected cells is crucial to induce strong immune responses via DNA vaccination, and that antibody responses towards a particular antigen could be significantly improved by increasing the secretory properties of the immunogen. The importance of antigen secretion in DNA vaccination has been addressed by other studies as well^{395,396,443}.

Despite the inclusion of an N-terminal secretory leader peptide to guide the translocation of DIII into the ER, significant protein secretion was only observed when the dimerising γ CH3 domain was fused to the C-terminal end of DIII, and was further improved after optimising the DIII coding sequences for expression in mammalian cells. The inclusion of the γ CH3 domain was critical for the efficacy of the vaccine, as it led to a significant increase in protein secretion by allowing intracellular transport after translocation into the secretory pathway. Although these modifications were relevant for all DIII constructs, the effect on 2DIII and 4DIII antigens was particularly important since the secretion of the viral encoded domains was barely detected. Surprisingly, the inclusion of the γ CH3 not only increased the immune response against the DIII antigen (in terms of anti-DIII titres measured in ELISA), but also enhanced the quality of the induced antibodies, as revealed by comparison of avidity indexes of sera from animals vaccinated with and without γ CH3.

Out of the several delivery strategies available for genetic vaccines, we decided to immunise mice with biolistic-delivered plasmid DNA. Our results confirm that gene-gun technology is an efficient way to induce strong antibody responses with very low amounts of total DNA: classical intramuscularly delivered genetic vaccines employ up to 100 μ g of DNA per dose^{460,464,466,469}, while our protocol required only 1 μ g (2 μ g for the tetravalent formulations) and was able to induce high antibody titres nonetheless.

It is particularly relevant to address the benefits of the conformational ELISA protocol implemented when discussing the results of the DIII- γ DNA vaccines. We took special care into designing the assay in order to properly evaluate immune responses. As pictured in Fig. 74, using the BAP/BirA system, the different proteins were obtained exclusively as mono-biotinylated products secreted from mammalian cells, and did not require any further purification since dialysed cultured supernatants were directly used as

a source of antigen and captured on avidin-coated plates. To ensure that equal amounts of protein were applied each time, the different antigens were normalised by western blot (using HRP-conjugated streptavidin). Thus the native structure and properties of the viral proteins were maintained and enabled us to determine the ability of the induced antibodies to recognise conformational epitopes, a feature commonly related to strong neutralising capacity³¹⁰.

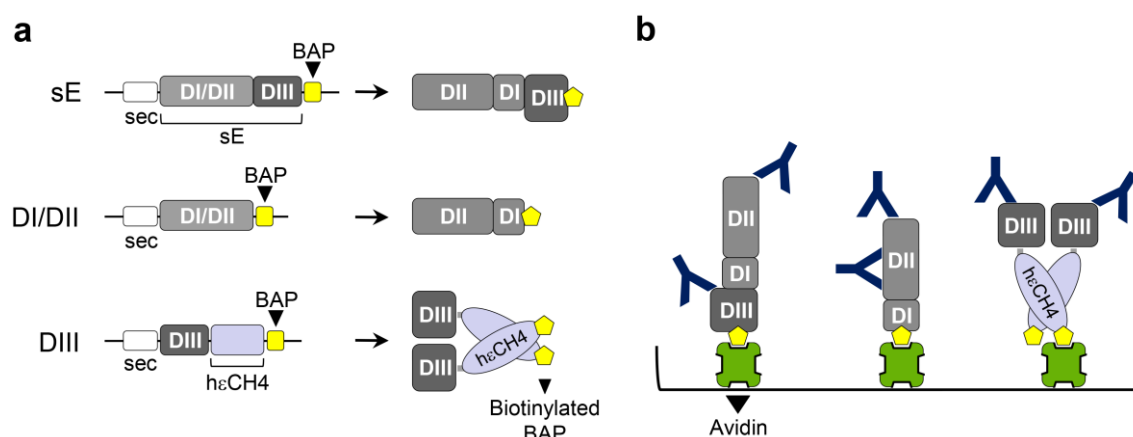


Figure 74. Conformational ELISA for detection of antibodies against E-derived antigens. a) Schematic representation of constructs used with the BAP/BirA system for obtaining mono-biotinylated sE, DI/DII and DIII proteins secreted from mammalian cells and used in the conformational ELISA. **b)** Scheme of the ELISA, with avidin-coated plates to equally capture biotinylated sE, DI/DII and DIII.

Different variations of ELISA are commonly used when evaluating new vaccines; however, the lack of standardised assays represents a significant problem when considering data reproducibility and reliable comparisons between studies. Since the amount of antigen used in our conformational ELISA has been normalised, all the results are comparable between them regardless of the nature, size or complexity of the protein used as antigen. Moreover, since our protocol was optimised and kept unchanged from the beginning of the project, the ELISA data obtained from mono-biotinylated antigens is highly reproducible. Given the impossibility to properly compare our results with previously published dengue vaccine studies, we decided to express the reactivity of the induced antibodies in terms of estimated antibodies concentrations. The antibody quantification was done by interpolating the OD value of different sera dilutions within a calibration curve of mAb 4G2 measured on 3sE. As mAb 4G2 is widely available and recognises a highly conserved epitope, it could be easily implemented as a reference to allow comparisons.

As shown in the results of our first round of vaccinations with DIII- γ candidates, all four antigens induced strong immune responses as revealed by the high antibody titres (and estimated concentrations) determined in ELISA. Of note, anti-2DIII responses were the strongest, while there were no significant differences between titres induced by 1DIII, 3DIII and 4DIII constructs against their homologous serotypes. Anti-DIII responses also

proved to be highly specific towards the homologous DIII antigens with very reduced cross-reactivity to the other serotypes, thus confirming the relatively high variability of DIII among DENV serotypes²⁷¹. Anti-3DIII antibodies, however, showed higher cross-reactive activities when tested on all four antigens, especially towards 2DIII and 4DIII. Moreover, the results demonstrate that anti-DIII antibodies were mainly directed to conformational epitopes readily exposed on the infective virion, as they strongly recognised the captured virus and the native DIII (and sE) antigen in ELISA, with only minimal reactivity towards equivalent amounts of denatured DIII proteins.

The results also suggest a correlation between antibody titres induced by the different DNA constructs and their neutralising capacity measured in PRNT₅₀ assays, since anti-2DIII antibodies showed significantly higher neutralising titres than the other groups, while there were no significant differences between the neutralisation induced by 1DIII- γ and 3DIII- γ against their homologous DENV serotypes. The anti-4DIII response was the exception as it showed significantly lower PRNT₅₀ titres than anti-1DIII and anti-3DIII sera despite their similar titres in ELISA. This appears to be characteristic of the 4DIII domain as presented in our vaccine format since DNA immunisations with a DIII- γ construct derived from a different strain (and genotype) of DENV4, led to essentially equivalent responses. In contrast, experimental data from previous studies indicate that variation between genotypes within each DENV serotype could impact vaccine efficacy significantly^{28,461,477-479}. For instance, failure of the CYD-TDV vaccine to protect against DENV2 (only 9.2% efficiency) during the Phase 2 study in Thailand, was thought to be due to differences in the circulating genotype³⁶⁵. Considering that the alternative 4DIII antigen belonged to same viral strain used in the neutralisation assay, our results suggest that DIII differences between the isolates used for vaccine design and evaluation, at least in our case, do not influence the neutralisation results of the study; further studies are needed to established if this is true also for the other serotypes and their respective DIII domains. In any case, if this effect is demonstrated, genetic vaccines, and particularly our DIII- γ platform, are highly adaptable and can be easily modified to take these elements into account.

It has been proposed that, in flaviviruses, neutralisation is achieved after the number of antibodies bound to the virus surpasses a determined threshold^{306,307}. In this way, binding of antibodies that target highly accessible epitopes, like the ones on DIII, is favoured and neutralisation can be achieved at low antibody concentrations as only a reduced occupancy of the available epitopes is required³¹⁰. Besides epitope accessibility, the neutralisation threshold is also dependent on antibody avidity²⁷². Indeed, recent studies have shown a positive correlation between antibody avidity and *in vitro* neutralisation for monoclonal antibodies against flaviviruses and other viral

infections^{28,307,309,477,480,481}. Recently, this correlation was also demonstrated in sera of DENV-infected patients⁴⁸². Our results confirm that DIII- γ immunisations induced high avidity antibodies in vaccinated animals, and that the avidity indexes were better predictors of the neutralising activities of sera when comparing samples with similar antibody titres: anti-3DIII and anti-4DIII sera, for example, were not significantly different in ELISA, but anti-3DIII antibodies showed a significantly higher avidity than the anti-4DIII sera, which correlated with the better performance of the 3DIII- γ vaccine in neutralisation assays. Thus, our results support the inclusion of avidity measurements, together with estimations of antibody titres and neutralising potential, into the preliminary evaluation of vaccine candidates, to better understand the behaviour of the induced antibody responses⁴⁸².

As mentioned before, antibodies exert their neutralising potential either by blocking viral interaction with cell receptors, or promoting the detachment of virus from the cell membrane, or by preventing the membrane fusion process following clathrin-mediated endocytosis. Even though we successfully proved the neutralising nature of anti-DIII antibodies induced by the DIII- γ vaccine, their mechanism of action remains an open question.

We believe that a combination of factors explain the efficacy and performance of the DIII- γ DNA vaccine. Intradermal, gene-gun delivery of plasmid DNA is particularly well-suited to target keratinocytes present at the immunisation site⁴⁸³. These cells show not only a high transfection efficiency, but are also able to modulate the immune response in the skin by staying in close interaction with immune cells of skin (such as Langerhans cells and intradermal lymphocytes) and producing a wide range of CKs which allows for proper presentation of the secreted antigen⁴⁸⁴. In the context of the DNA vaccine, this leads to both Th1/Th2 immune responses, contrary to the classical intramuscular injection of DNA, which mainly activates the Th1 pathway⁴⁸⁵. Increased antigen availability, achieved by optimising the secretory profile of the DIII proteins, was also critical since codon-optimised DIII- γ constructs induced immune responses that were significantly stronger, both in terms of antibody titres and neutralising capacity, than those elicited by the non-optimised (and less secreted) DIII- γ antigen, which were stronger than those from mice vaccinated with the non-optimised DIII alone. Furthermore, the γ CH3-mediated dimerisation of the DIII antigen, could have also contributed to the immunogenicity of the vaccine, as it would favour engagement of the B-cell receptor (BCR) and subsequent activation of naive B cells. In addition, the xenogeneic nature of the γ CH3 domain may have induced further activation of T helper cells⁴⁸⁶; however, since the study was focused on the analysis of the antibodies elicited by the vaccine, the cellular factors were not specifically addressed. Together, when considering the contribution of the modifications

introduced to the DIII antigen as part of the vaccine design process, our data confirms that codon optimisation significantly improved antibody titres by enhancing protein secretion from transfected cells, while the inclusion of the γ CH3 domain increased immunogenicity and secretion levels of the antigen, as revealed by the avidity index and neutralising capacity of the induced antibodies.

As a proof of principle, we designed a tetravalent DIII formulation using a combination of the plasmids coding for the different DIII- γ constructs. Anti-DENV immunity was efficiently induced by the tetravalent formulation; however, immune responses were considerably lower in terms of antibody and PRNT₅₀ titres when compared against the four monovalent vaccines, most likely a reflection of the reduced amount of DIII-specific plasmid used for vaccination. Notably, for the tetravalent formulation, neutralisation activity was strongest against DENV3, while PRNT₅₀ titres towards DENV4 were further reduced. Avidity indexes measured against each DIII protein were lower than those obtained when evaluating monovalent vaccines in all cases, even though this value could be misleading due to the cumulative effect of the cross-reactive antibodies present in the tetravalent sera. Together, the data obtained from the tetravalent formulation indicated that further development and optimisation of the DIII- γ vaccine was needed to reach values comparable to those of the monovalent formulations.

The enhancement in DIII- γ secretion after changing the expression vector used for DNA vaccination was surprising. As explained before, the FDA-approved pVAX1 vector replaced the pcDNA3.1 backbone as part of the optimisation of the DIII- γ vaccine in order to simplify the transition to an eventual clinical trial and augment the significance of our experimental data. However, upon preliminary evaluation of the new expression platform, it became evident that the secretion of the same DIII- γ encoded proteins increased. These results could be explained by accounting for the size differences between both vectors, since pVAX1 is considerably smaller (5.428 vs. 2.999 nucleotides for the empty pcDNA3.1 and pVAX1 plasmids respectively) given that all sequences not required either for plasmid replication in bacteria, or protein expression in mammalian cells, were removed from it in order to comply with FDA requirements⁴⁷¹; as a result, pcDNA3.1-encoded DIII- γ plasmids are almost 40% bigger than their pVAX1-equivalents, which means that the number of plasmid units transfected (and consequently vaccinated) when using the same mass of DNA is higher for the pVAX1-encoded protein. Since the differences in secretion were not equal among the four DIII proteins, other vector-related factors could also be involved in the secretory enhancement of DIII- γ protein; however, we did not perform any additional investigation on the subject. Independently of the cause, this is a highly desirable feature

due to the positive correlation demonstrated between antigen secretion and genetic vaccination.

The decision to evaluate different vaccination protocols and delivery alternatives was made in an attempt to further optimise the DIII- γ vaccine. AAV vectors have been largely evaluated in gene therapy and are increasingly becoming attractive vaccine carriers to mediate DNA delivery in genetic immunisations. In fact, several AAV-vectored DNA vaccines against pathogens like Influenza virus, Human Papilloma virus (HPV) and HIV have been described⁴¹⁵. In the case of dengue however, only one AAV-vectored vaccine has been reported, with modest results⁴²².

Our results indicate that both AAV serotypes employed were able to efficiently deliver the plasmids to intradermal cells of vaccinated animals, as evidenced by the detection of anti-DIII specific antibodies in all groups; however, induction of antibody responses was more efficient in AAV6 than AAV9, and none of the examined groups were able to measure up to our initial 3-dose gene-gun strategy, in terms of both antibody titres and neutralising capacity of the induced sera. Notably, our data confirmed AAV6 preferred tropism for muscle cells, as the subcutaneous inoculation (to target keratinocytes) of the virus was poorly immunogenic while the intramuscularly administration was the second best protocol analysed. In addition, protocols involving combinations of gene-gun and AAV strategies were successful in eliciting stronger antibody responses with a simplified vaccination schedule that would be more easily implemented in a clinical setting. It was unfortunate that a GG+AAV6_{IM} group was not included in the assay, since the results suggest that it could have elicited responses closer to those of 3xGG vaccinated mice; future experiments should address this question. Collectively, our data indicate that genetic vaccination using a gene-gun mediated delivery platform is superior to vaccination protocols involving AAV. Indeed, together with high manufacturing costs and the need to employ high titre doses, low immunogenicity has been one of the major drawbacks for the development of AAV-vectored vaccines⁴¹⁵. However, future projects should seek to improve AAV vaccination efficiency given their significant benefits in the context of dengue; in particular, the possibility of developing an effective single-dose vaccine would be highly suitable not only for implementation in the clinical setting, but also to facilitate mass vaccination of vulnerable populations. Proper evaluation of vaccination protocols involving different AAV serotypes, combination with novel adjuvants and heterologous prime/boost strategies, would provide valuable additional data.

In addition to strong neutralising protection towards the four DENV serotypes, the efficacy of a vaccine also relies on its safety and capacity to induce persistent protective immunity over prolonged periods of time. Indeed, the WHO recommends verifying that the vaccine-induced antibody responses do not lead to increased risk of severe disease due

to waning antibody titres in time⁴⁸⁷. For this reason, we decided to evaluate the immunogenicity of the pVAX1-encoded antigens in mice over relatively long-periods of time. Our results showed that monovalent antibody titres were considerably higher in these groups than in the pcDNA3.1-vaccinated mice, probably reflecting the enhanced secretory profile of the DIII- γ within the pVAX1 vector. Notably, the immune responses remained high and stable over the follow-up period both on terms of antibody titres and relative avidity. In agreement with our preliminary vaccine evaluations, the four types of anti-DIII sera were highly specific and mainly recognised the homologous DIII antigen; as before, anti-3DIII sera showed the highest cross-reactivity. However, as a result of the enhanced homologous responses, the differences between homologous and heterologous activities increased. Besides long-term stability, the antibody titres were highly balanced when compared amongst them, in contrast to the results obtained in the first round of vaccinations and further confirming the improvement of the DIII- γ vaccine.

As expected from the higher antibody titres, the neutralising capacity of each group of sera, as measured by the FRNT₅₀ tests, was also higher; notably, the new 4DIII- γ formulation, whose secretion increased the most when switching to the pVAX1 backbone, showed DENV4 neutralisation titres ≈ 4 times higher than the ones measured initially. It should be noted that the introduction of the FRNT₅₀ assay instead of the classical PRNT₅₀ could have influenced the results and at least part of the observed differences might derive from methodological factors and not to actual improvements of the DIII- γ vaccine; however, the differences seen for anti-2DIII (≈ 2 times higher), anti-3DIII (>2 times higher) and anti-4DIII could not be explained by a modification in the read-out approach alone.

After an infection, anti-DENV antibodies start to decline rapidly until reaching levels that remain stable for years²²⁰. It has been suggested that these rapidly declining antibodies are not selected for expansion due to high cross-reactive activity and weak binding to the viral particle, while the stable level of remaining antibodies comprise, on the contrary, strongly neutralising type-specific antibodies⁴⁸⁸. The increase in the FRNT₅₀ titres over the one-year follow up are difficult to explain: even though the results suggest a maturation-related enhancement of the immune response induced by the DIII- γ , the stability of the antibody titres, the specificity of the responses and the avidity of the sera remained unchanged. Small increases of potent neutralising antibodies as part of the evolution and selection of the immune response could have a significant impact on the neutralisation assays but not on the other tests as they would be masked by the high concentration of antibodies. Adsorption studies using heterologous antigens to remove cross-reactive, and likely poorly-neutralising, antibodies may help to evidence subtle differences in the behaviour of highly specific antibodies over time; however, even at their lowest, the neutralising capacity of these sera was significantly better than the previous

pcDNA3.1 vaccines, and considering that the main focus of these experiments was the optimisation of a tetravalent DIII- γ formulation, we did not investigate this behaviour in detail.

One of the main problems observed during the preclinical studies of CYD-TDV vaccines was the apparent immunological interference between DENV serotypes which resulted in an unbalanced immune response dominated by some serotypes. In an attempt to mitigate this effect they implemented several immunization approaches including i) sequential and simultaneous administration of different and complementary bivalent formulations, ii) pre-immunisation with different flaviviruses and iii) reformulation of the vaccine dosage based on the detected immunodominance⁴⁸⁹. In our case, we implemented a similar approach by evaluating different and complementary bivalent formulations of our DNA vaccine in attempt to obtain an immune response evenly distributed among the four DENV serotypes in mice. As with the monovalent formulations, tetravalent formulations of the pVAX1-encoded DNA vaccine also showed enhanced antibody titres against all DIII epitopes when compared to the results of the first tetravalent immunisation. Similar to the first round of vaccination, antibody responses were unbalanced among the different DIII antigens with all formulations showing higher reactivity towards 1DIII soon after vaccination; however, immune responses balanced in time even though anti-3DIII responses were consistently lower for all DNA-mix formulations. Indeed, compared to tetravalent formulations using the DNA-mix approach, the antibody responses obtained with the Beads-mix preparation were better balanced. This could reflect a potential benefit of inducing expression of a single DIII- γ antigen per transfected cell (assuming that each cell is transfected by only one DNA-coated carrier) as each gold particle was treated with a single plasmid as opposed to the DNA-mix formulations, where each transfected cell would express, at least, two different DIII proteins simultaneously. Unexpectedly, results of the FRNT₅₀ performed on each DENV serotype did not reflect the balanced response seen in ELISA and were distributed similarly to the neutralisation responses observed during the first tetravalent test. Since this behaviour appeared to be independent of the formulation analysed, the results probably reveal the properties of the anti-DIII response when all DIII- γ antigens are equally administered in mice, thus, future evaluations where the amount of each DIII plasmid follows the ratio of the FRNT₅₀ titres seen here, would provide important data towards the development of a formulation with balanced neutralising activity. Notably, the neutralising activities of the tetravalent sera differed from the monovalent vaccines in two main points:

1. Except for strong enhancement in DENV1 neutralisation, FRNT₅₀ titres against the other three DENV serotypes did not show any major differences when compared against the initial pcDNA3.1 results. The improvement in DENV1 neutralisation was seen only for the Beads-mix preparation, further supporting the benefits

attached to this formula.

2. Neutralising activities against the four DENV serotypes remained stable over the one-year study as FRNT₅₀ titres measured at months 1 and 12 after vaccination were not significantly different.

Collectively, even if the mouse immunisation model poorly reproduces the human immune responses against dengue, the results obtained from these longitudinal analyses provide important information that could be used to further improve the vaccine. Specifically, the data suggest that evaluation of DIII- γ tetravalent vaccines with increased dosages of DNA could match the responses measured in mice vaccinated with single antigens; in addition, due to the unbalanced neutralising titres obtained repetitively when using equal amounts of each DIII- γ plasmid, future experiments should implement a FRNT₅₀ titre-based normalisation approach when determining the ratio of constructs included in the tetravalent vaccine formulations.

The use of DIII in the development of vaccines against Dengue has been previously reported for recombinant protein subunit vaccines^{378-385,388,490}, DNA vaccines^{398,408,465} or viral-vectored vaccines^{463,491}. Our DIII- γ vaccine is different as the antigen was carefully designed to increase immunogenicity. Indeed, when compared to candidates evaluated using similar methodologies, the DIII- γ DNA vaccines elicited higher neutralisation titres against all four serotypes, even when considering protocols implementing heterologous prime/boost strategies using a combination of proteins and DNA^{379,408}. Significantly, the evaluation of ADE induced by DIII-specific sera on THP-1 cells demonstrated that the highly specific immune responses elicited by the DIII- γ DNA vaccine mainly promote the infection of the homologous serotypes, with reduced effect over the heterologous ones therefore; ADE would be driven mainly by the decay of homologous antibody titres to sub-neutralising levels. However, given the poor reproducibility of the mouse model in the case of dengue, the conclusions that can be extracted from this study regarding the efficacy of the vaccine in NHPs or humans, are very limited and require further assessment. These results also highlight the need for further optimisation and analysis of the tetravalent formulation to ensure not only the strength and balance of the response, but also to measure antibody longevity and reduce eventual risks of ADE.

Even if anti-DIII antibodies have been shown to have higher neutralising capacity than those directed against epitopes on DI and DII²⁷¹, most DENV vaccine candidates continue to use the whole E protein as their main immunogenic component³³⁶. This is of relevance as data indicate that the immune response against E is heavily dominated by epitopes on DII, especially those located in the vicinity of the FL^{279,492}. Taking this into account, we compared DIII- γ and sE-induced responses using the gene-gun platform. The results

clearly showed that immune responses to the better secreted DIII- γ were significantly stronger than those obtained from animals vaccinated with whole sE. DI/DII immunodominance was evidenced by the significantly different reactivity of anti-sE sera against DI/DII (strong) and DIII (weak) antigens. This was further confirmed after comparing the reactivity profile of anti-DI/DII and anti-sE sera obtained after vaccinating with plasmids derived from DENV3 and DENV4. As a result, the neutralisation titres induced by the DIII- γ construct were also significantly higher than those observed in sE-vaccinated animals. Indeed, when measured against other (methodologically comparable) DENV E-based DNA vaccines, the DIII- γ constructs induced stronger neutralisation titres in vaccinated mice despite the low amount of DNA used for immunisation^{460,464,466,468,469}. Furthermore, the neutralising response towards the DIII- γ candidate was higher than other DNA vaccines in which the sE antigen was further modified to enhance immunogenicity^{390,409,462,467,493,494}.

Even though the possibility of ADE among heterologous flaviviruses has been known for many years^{322,323}, it was only recently that the close immunological relationship between DENV and ZIKV was described by demonstrating that poorly-neutralising cross-reactive antibodies against either virus are able to enhance infection of the other^{324,444,495}. In fact, studies using mAbs isolated from DENV or ZIKV infected patients, showed *in vivo* and *in vitro* enhancement of ZIKV and DENV, respectively^{287,444}. DENV and ZIKV are closely related as evidenced by the high degree of homology shared between their respective E proteins (between 54-59% when comparing ZIKV against all four DENV serotypes^f) explaining the cross-reactivity revealed by these studies^{27,287}. In addition, the overlap of DENV-endemic areas and those involved in the current ZIKV outbreaks has raised concerns regarding the possible effect of pre-existing DENV immunity in ZIKV pathogenesis³²⁴. The same reasoning could also apply to other flaviviruses that show cross-reactivity with DENV, such as WNV and YFV³²³. These new data have significant repercussions for vaccine research, as they raise the additional need to consider infection enhancing properties of the induced antibodies not only against the different serotypes of DENV, but against other flaviviruses as well.

Considering this, we decided to compare the ADE and neutralisation profiles of polyclonal sera obtained from mice vaccinated with plasmids encoding DIII or DI/DII from different DENV serotypes, among different flaviviruses. In contrast to the broad cross-reactivity of anti-DI/DII sera (and anti-sE sera in consequence, due to DI/DII immunodominance), the lack of cross-reactivity of anti-DIII antibodies against other flaviviruses further confirmed the specificity of DIII epitopes.

^f For context, the aminoacid sequence of E protein differs by 30-35% among the four DENV serotypes.

As expected, anti-DIII antibodies induced by the monovalent or tetravalent vaccine formulations, showed no neutralising activity nor, notably, enhanced the infection of WNV, ZIKV and YFV, thus confirming the DENV specificity of the DIII- γ vaccine. On the other hand, both anti-DI/DII sera induced relatively weak neutralisation of DENV serotypes, but did not neutralise and strongly enhanced WNV, ZIKV and YFV infection in Fc γ -receptor bearing cells. This is an important feature to consider as it has been described that ADE capacity is present even in strongly neutralising antibodies³⁰⁹; now, our data indicate that vaccination with DI/DII epitopes contribute almost exclusively to ADE with no neutralisation.

In agreement with previous studies, our data confirm that highly cross-reactive antibodies, especially those against DI/DII epitopes, are the main drivers of ADE not only among DENV serotypes²⁷⁸, but also among other flaviviruses like ZIKV, WNV or YFV^{323,444}. Moreover, a recent study with mAbs isolated from mice immunised with ZIKV E protein, showed that highly-neutralising, type-specific antibodies were mainly directed to DIII epitopes, suggesting that ZIKV DIII would be a safe immunogen in vaccine formulations⁴³⁷.

Our results complement the information extracted from the study of monoclonal antibodies and also provide much needed data regarding the functions and characteristics of domain-specific polyclonal antibodies, which are critical to properly understand the natural immune response following immunisation. Indeed, most of what is known about the immune response against flaviviruses comes from the study of mAbs; however, the reality of polyclonal responses is significantly different as it comprises a mixture of antibodies that recognise several epitopes, have different neutralising potency and cross-reactive features³¹⁰. In this context, our experiments represent one of the first domain-specific descriptions of the antibody response induced against DENV E protein. Together, the results suggest that DIII could be used to develop a tetravalent DENV-specific vaccine avoiding cross-reactivity with other flaviviruses.

The recent description of conserved complex quaternary epitopes in DENV and ZIKV, able to induce strong cross-neutralising antibodies, has opened the possibility for the development of universal effective vaccines against both viruses^{287,289}. However, there are serious concerns regarding them as viable vaccine candidates in the near future, since their structure is largely composed by DI/DII determinants, which would, by virtue of their immunodominant nature, induce poorly-neutralising cross-reactive responses. In this scenario, the effective use of cross-neutralising epitopes would require, in theory, either the isolation of the specific conformation of the epitope, or the removal of the other immunogenic epitopes on the surface of the E proteins, which are both highly complex tasks.

There are limitations to these conclusions that should be considered: As detailed in the introduction, there is abundant evidence supporting the effect of antibody-mediated enhancement and disease between dengue serotypes; however, there are no reports confirming a correlation between *in vitro* ADE assays and increased risk of infection by other flaviviruses; moreover, there is no clinical evidence of increased disease severity from WNV, ZIKV and YFV in dengue-immune patients. Nonetheless, even though the full influence of ADE during Flavivirus infection has not been determined, the risk for haemorrhagic complications found in (CYD-TVD)-vaccinated children suggests that these concerns are relevant and should be kept under consideration during the development of new vaccines, where proper antigen selection and design is of paramount importance.

In conclusion, the results presented in this section explain the efficacy of the DIII- γ DNA vaccine in four key points:

1. The use of the DNA vaccination allowed us to produce the antigen in a way that effectively surrogates the viral infection process, thus enabling the induction of stable and long-term immune responses.
2. The design and engineering of the immunogen to maximise antigen availability, by introducing the dimerising γ CH3 domain and codon-optimisation, significantly increased protein production and secretion, favoured antigen presentation to immune cells and improved the quality of the induced antibodies.
3. The selection of DIII, instead of the whole E, as the main immunogen produced DENV-specific strongly-neutralising responses, reducing the level of cross-reactivity between DENV serotypes and the risk of ADE toward other flaviviruses.
4. By targeting the intradermal compartment using gene-gun technology, plasmid DNA can be delivered to keratinocyte cells within the epidermis that are able to produce and secrete the encoded antigen in an immunologically favoured environment^{483,484}.

The results discussed in this section support the idea of further developing biolistic-delivered DNA-vaccines focused on DIII to induce strong, stable and type-specific neutralising antibodies that circumvent the risk of ADE among flaviviruses.

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